

# Rapid Antibiotic Susceptibility Testing by Deuterium Labeling of Bacterial Lipids in On-Target Microdroplet Cultures

Evan A. Larson, Josiah J. Rensner, Kristina R. Larsen, Bryan Bellaire, and Young Jin Lee\*



Cite This: *J. Am. Soc. Mass Spectrom.* 2022, 33, 1221–1228



Read Online

ACCESS |



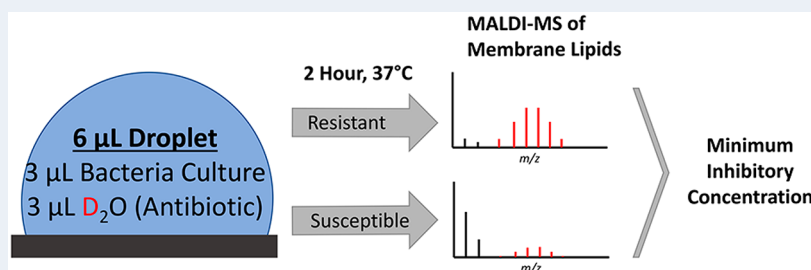
Metrics & More



Article Recommendations



Supporting Information



**ABSTRACT:** Antimicrobial resistance is a serious challenge facing human and veterinary health. Current methods of detecting resistance are limited in turn-around time or universal detection. In this work, a new antimicrobial susceptibility test is developed and validated, which utilizes deuterium labeling of membrane lipids to track the growth of bacterial cells. We hypothesize that deuterium uptake and subsequent labeling of lipids can be detected using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Additionally, bacteria growth is performed on the MALDI target, minimizing sample preparation materials and time. When two *Escherichia coli* strains are grown in the presence of deuterium oxide, labeling can be detected in as little as 30 min to 2 h. The labeling efficiency, or the ratio of labeled to unlabeled lipid peaks, provides information about the growth rate of bacteria. This growth ratio can differentiate between resistant and susceptible strains of bacteria as a resistant strain will maintain ~50% labeling efficiency between untreated and treated cultures. In comparison, a susceptible strain will see a decrease in fractional abundance of deuterium from ~50% in the untreated to ~10% in the treated. This approach is applied to measure the minimum inhibitory concentration (MIC) of the resistant and susceptible strains from on-target microdroplet culture in a range of antibiotic concentrations. The first antibiotic concentration with a significant decrease in fractional abundance of deuterium correlates well with a traditionally obtained MIC using broth dilution, indicating the clinical relevance of the results.

**KEYWORDS:** deuterium labeling, antimicrobial resistance, MALDI, minimum inhibitory concentration, antibiotic susceptibility test, *E. coli*, membrane lipids

## INTRODUCTION

Antimicrobial resistance and the emergence of multi-drug-resistant bacteria is a growing concern for human and veterinary healthcare. Antibiotic misuse and overuse have led to highly resistant bacterial strains, including ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter*) pathogens, which are dangerous and difficult to treat.<sup>1,2</sup> As new antibiotic development slows, it is imperative to develop new methods to diagnose and determine treatment for infections quickly and accurately in a clinical setting. Antibiotic susceptibility tests (ASTs) are commonly used in clinical settings to determine the minimum inhibitory concentration (MIC) of an antibiotic for a particular strain.<sup>3–5</sup> The MIC provides the minimum concentration at which the antibiotic inhibits the growth of a strain of a bacteria and gives a reference for the resistance of that strain. The most commonly used methods, such as broth dilution or agar dilutions, can provide MIC information regardless of the

resistance mechanism but suffer from long culture times, leading to a slow turn-around time (TAT) from diagnosis to treatment.<sup>6</sup> Methods such as polymerase chain reaction (PCR) amplification of resistance genes or immunoassays can rapidly identify known resistance genes. These methods rely on databases that may not be comprehensive and provide no MIC information.<sup>7,8</sup> The ideal clinical AST method should have a fast TAT, function regardless of the resistance mechanism or bacterial strain, and be simple and high-throughput.

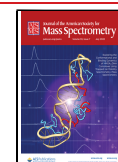
Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has become widely available in clinical

**Received:** February 21, 2022

**Revised:** May 16, 2022

**Accepted:** May 16, 2022

**Published:** May 27, 2022



laboratories, especially MALDI-time-of-flight (TOF) systems such as the MALDI Biotyper<sup>9,10</sup> or Vitek<sup>11</sup> systems to identify bacterial strains. These MALDI-TOF systems are high-throughput with short analysis time and high sensitivity, allowing for rapid identification of species or strains based on their protein fingerprint. However, on their own, these systems provide no information about resistance or MIC, only detecting the species or strain present. MALDI-MS has been recently used for antimicrobial resistance detection beyond bacterial identification. Idelevich et al. developed an AST using an on-target microdroplet culture of bacteria to detect cell growth by protein fingerprinting, which successfully identified resistant strains from agar cultures and directly from blood cultures.<sup>12–15</sup> The Goodlett and Ernst groups have developed on-target extraction and detection of membrane glycolipids, specifically lipid A, to classify Gram-negative bacterial strains and identify colistin resistance.<sup>16–19</sup> Additionally, they have cataloged membrane glycolipids to fingerprint ESKAPE pathogens and their resistance.<sup>20,21</sup> Zhang et al. identified metabolic biomarkers to identify extended-spectrum  $\beta$ -lactamase-resistant *E. coli* strains.<sup>22</sup> Other studies have focused on detecting hydrolysis products of  $\beta$ -lactam drugs such as carbapenem, which can be detected very quickly.<sup>23,24</sup> Currently, there is no clinical method that takes advantage of the high-throughput and sensitivity of MALDI-MS to detect antimicrobial resistance and determine the MIC of a strain.

It is challenging to develop a rapid AST typically limited by the culture time in response to antibiotics. Some automatic tools are developed based on microscopic physiological changes and approved by the Food and Drug Administration, but they still require 4–16 h of turn-around time.<sup>6</sup> Stable isotope labeling can rapidly track metabolic changes as organisms grow. Berry et al. tracked the incorporation of deuterium labels using Raman spectroscopy to quickly identify and sort bacteria in a mouse cecum sample.<sup>25</sup> Tao et al. used Raman spectroscopy to track deuterium incorporation to determine MIC.<sup>26</sup> Kopf et al. tracked pathogen growth rate in cystic fibrosis patients using isotope ratio mass spectrometry.<sup>27</sup> Neubauer et al. developed an extraction method for deuterium-labeled lipids and used deuterium incorporation to measure bacterial growth.<sup>28</sup> Incorporation of the D-label into newly synthesized lipids can be easily detected by mass spectrometry and indicates that bacteria are growing, dividing, and producing new lipids. Additionally, deuterium labeling is a straightforward experiment, and simply growing bacteria in the presence of D<sub>2</sub>O will rapidly detect newly synthesized lipids.

Here, we propose using stable isotope labeling of membrane lipids, specifically deuterium labeling, to track the growth of bacterial strains in the presence of antibiotics using MALDI-MS to determine resistance or MIC. We combine it with the on-target microdroplet culture method developed by the Idelevich group<sup>12</sup> to simplify the sample preparation by culturing and preparing the sample for MALDI analysis on a single plate. However, instead of typing bacteria using protein signals, which requires a minimum of 4 h, we measure a minute amount of deuterium labeling in membrane lipids, which dramatically reduces the culture time. In this proof-of-concept work, a model *E. coli* system was used with two strains, JJ1886 and MG1655, which are resistant and susceptible to ciprofloxacin, respectively.

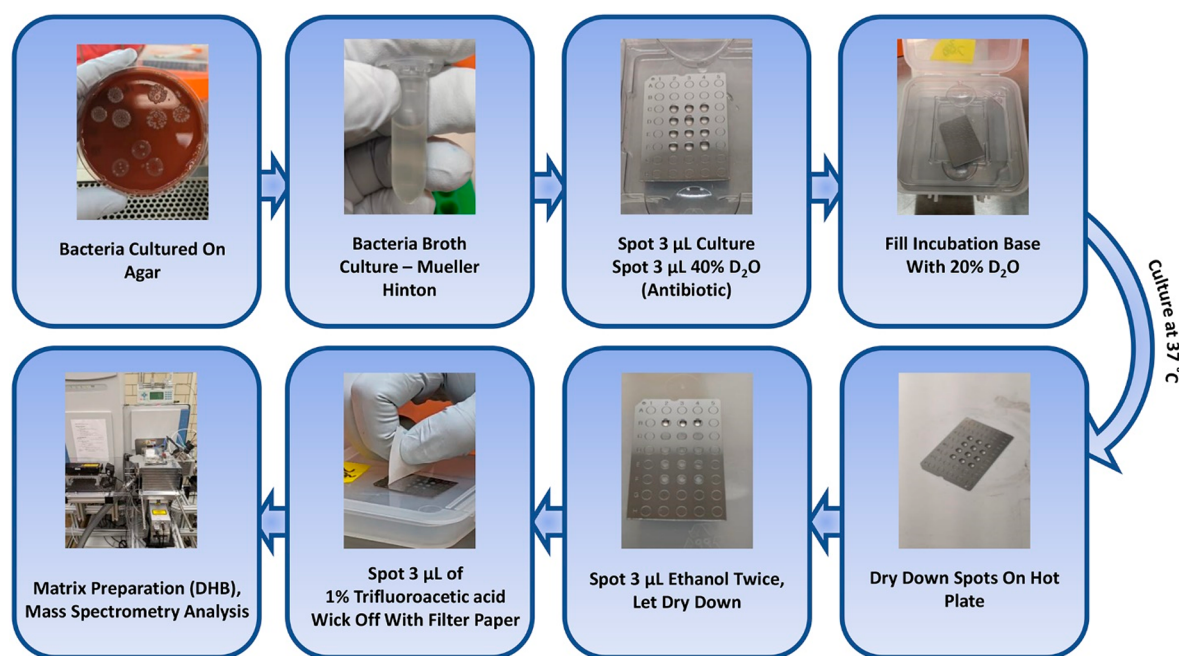
## METHODS

**Microdroplet Bacterial Culture.** Two *Escherichia coli* strains, MG1655 (MG) and JJ1886 (JJ), were removed from  $-80$  °C storage and streaked onto a Columbia blood agar plate. The initial culture was made by incubating the agar plates overnight in a 37 °C incubator to grow the initial bacteria stock. After overnight incubation, each bacteria strain was inoculated into 1 mL of Mueller Hinton broth. A colony-forming unit (CFU) value was determined for the initial stock using an OD600 measurement, and then the stock was diluted to a concentration of  $2 \times 10^8$  CFU/mL.

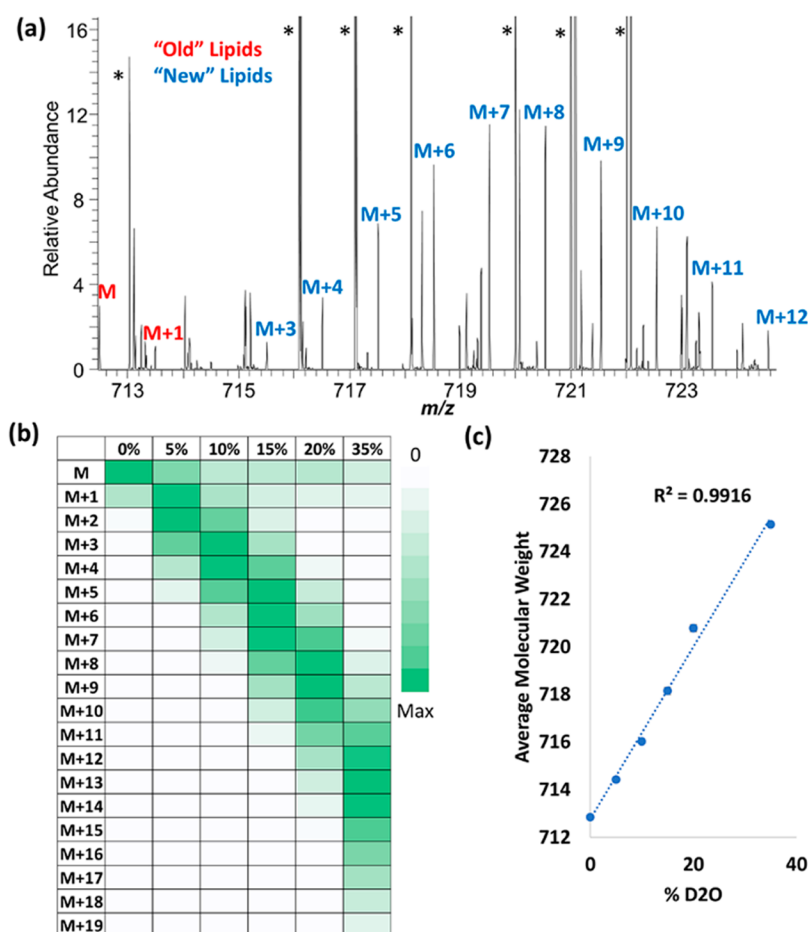
The base of the incubation chamber (Transport Box for MSP Biotarget, 8270006, Bruker Daltonics) was filled with 4 mL of D<sub>2</sub>O adjusted to the concentration used in the growth medium, typically 20%. A  $\mu$ Focus array plate (Hudson Surface Technology, Closter, NJ) was cut in half and placed into the incubation chamber. A 3  $\mu$ L volume of the broth culture of each strain was spotted onto the plate, and then 3  $\mu$ L of D<sub>2</sub>O was spotted on top of that, with or without ciprofloxacin. The final concentration of D<sub>2</sub>O on the spot was equivalent to the concentration in the base of the chamber. Each condition was spotted in triplicate on the plate, typically alternating between the two strains. After spotting, the plates were transferred to the incubator at 37 °C for 30 min, 1 h, or 2 h, depending on the experiment. After incubation, each plate was placed onto a hot plate ( $\sim 100$  °C) and dried until each culture spot was completely dry. To lyse the cells, 3  $\mu$ L of ethanol was spotted onto each culture twice and allowed to air-dry between and after each application. Afterward, 3  $\mu$ L of 1% trifluoroacetic acid (TFA) was spotted on each culture. After 30 s, the TFA was wicked off using filter paper, which removed the majority of the water-soluble broth contamination. Bacteria experiments were performed in a biosafety level 2 lab, and bacteria on the MALDI plates were inactivated using methods approved by the Institutional Biosafety Committee (IBC).

**Broth Dilution Experiment for Minimum Inhibitory Concentration.** After overnight culture on Columbia blood agar plates, both *E. coli* strains were inoculated into Mueller Hinton broth at a concentration of  $2 \times 10^8$  CFU/mL. A 50  $\mu$ L volume of each strain culture was then mixed with 50  $\mu$ L of ciprofloxacin for a range of concentrations (0, 1.25, 2.5, 5, 10, 20, 40, 80, 160, and 320  $\mu$ g/mL for MG and 40, 80, 160, and 320  $\mu$ g/mL for JJ) in 20% D<sub>2</sub>O and cultured overnight. After overnight culture, each culture was then diluted 10-fold seven times ( $10^0$ ,  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$ ,  $10^{-7}$ ). A 20  $\mu$ L aliquot of each dilution was spotted in triplicate on Columbia blood agar plates and cultured overnight. After overnight culture, the number of colonies formed for each dilution was counted and converted to CFU/mL. An additional broth dilution experiment was performed just for MG with a ciprofloxacin concentration range of 0, 0.039, 0.078, 0.156, 0.313, 0.625, 1.25, and 2.5  $\mu$ g/mL.

**Mass Spectrometry Analysis and Data Analysis.** After lysing and cleaning steps, 3  $\mu$ L of 30 mg/mL 2,5-dihydroxybenzoic acid (DHB) in 100% ethanol was spotted onto each culture and dried down. Plates were analyzed on a MALDI source (MALDI Injector; Spectrograph, Kennewick, WA) coupled to an Orbitrap mass spectrometer (QExactive HF; Thermo Fisher Scientific, San Jose, CA). The MALDI source uses a 349 nm laser (Explorer One, Spectra Physics, Milpitas, CA) at a 500 Hz repetition rate with an energy of  $\sim 4$   $\mu$ J. Data were collected in positive mode for the  $m/z$  range of



**Figure 1.** Sample preparation workflow for deuterium labeling AST experiment.  $\text{D}_2\text{O}$  concentration and culture time can be adjusted as needed.



**Figure 2.** (a) Mass spectrum of PE 32:1 after labeling with 20% deuterium for 2 h. Two main distributions of peaks are present, unlabeled "old lipids" (red) present at the start of the culture and labeled "new lipids" (blue) newly synthesized in on-target microdroplet culture. The \* indicates matrix or broth background. (b) Isotopologue distribution for PE 32:1 after 2 h cultures for a range of  $\text{D}_2\text{O}$  concentrations (0–35%). The labeled peak envelope is shifted based on the  $\text{D}_2\text{O}$  concentration. (c) Plot for the average molecular weight of PE 32:1 versus  $\text{D}_2\text{O}$  concentration.

680–850 with 120,000 resolution at  $m/z$  200 in profile mode. Once collected, ASCII data were extracted using QualBrowser (Thermo) from raw files. An in-house Python script was used to extract labeled lipid peaks and calculate the average molecular weight for each lipid. Extracted mass spectra in .csv format and the Python program used for data analysis are available from the corresponding author upon request.

## RESULTS AND DISCUSSION

**Method Development.** The adapted microdroplet method is shown in Figure 1. After overnight culture on an agar plate, the bacteria are scooped into a broth culture and adjusted to  $2 \times 10^8$  CFU/mL. A higher initial bacteria concentration was used in this work compared to that in the Idelevich work ( $1 \times 10^6$  CFU/mL) as this approach focuses on detecting a minute amount of deuterium labeling in lipids with a minimum culture time, and starting with an initial stock of unlabeled lipids provides a baseline of growth. This broth culture is then spotted in 3  $\mu$ L droplets on a stainless-steel microarray plate, and 3  $\mu$ L of D<sub>2</sub>O with or without ciprofloxacin is spotted on top. The plate is then cultured at 37 °C in a plastic incubation chamber adapted from a Bruker Biotyper plate transport container. The bottom part of the chamber contains 4 mL of D<sub>2</sub>O to maintain a humid atmosphere, which guarantees the droplet does not dry out during incubation. It is essential to ensure that the D<sub>2</sub>O concentration in the base of the chamber matches the concentration on the droplet, so the droplet concentration is not affected by water vapor exchange. After a 30 min to 2 h culture, the culture droplets are dried using a hot plate. This allows the cell lipids to adhere to the plate and not be lost in the subsequent steps. After drying down, 3  $\mu$ L of ethanol is spotted twice to lyse cells and free membrane lipids, and the spots are allowed to air-dry. After ethanol treatment, 3  $\mu$ L of 1% TFA is spotted and wicked off using filter paper after 30 s. This removes most Mueller Hinton broth interferences while leaving behind lipids. These two treatments were optimized based on lipid signals (data not shown). After treatment, matrix is applied, and the plate is analyzed on the mass spectrometer.

Typical labeled data are shown in Figure 2a for PE 32:1, a common *E. coli* membrane lipid, after a 2 h culture. With 20% D<sub>2</sub>O, the lipid peaks are separated into two isotope distributions, “old” lipids and “new” lipids. The “old” lipids consist of the unlabeled monoisotopic peak and single <sup>13</sup>C peak with the natural <sup>13</sup>C abundance. These peaks represent the lipids of bacterial cells present at the start of the culture. The new lipids consist of a Gaussian distribution of deuterium-labeled peaks, typically centered around  $M + 7$  or  $M + 8$  for 20% D<sub>2</sub>O. These peaks represent deuterium labeling of the fatty acyl chains of the phospholipid, which in turn is a metric for new growth in the microdroplet culture. The 1% TFA wash and the matrix application provide opportunities for the back exchange of readily exchangeable hydrogens (e.g., –OH) so that only carbon-bound deuterium will be measured, which improves the reproducibility of the data. The ratio between the new lipids (labeled) and the old lipids (unlabeled) provides a metric for the lipid turnover rate and therefore the growth and division of the bacteria in the microdroplet. Alternatively, calculating the average molecular weight of a particular lipid in different conditions provides a simple method for comparing lipid growth in those conditions, such as D<sub>2</sub>O concentration, antibiotic concentration, or differing strains. For example, in

three replicates for 20% D<sub>2</sub>O labeling, the average molecular weight of PE 32:1 is 720.7843 Da compared to that of an unlabeled control, which is 712.8373 Da.

### D<sub>2</sub>O Concentration Optimization and Lipid Selection.

D<sub>2</sub>O is lethal to complex organisms at about 20–40% of body water content,<sup>29</sup> but bacteria are amenable to higher concentrations, allowing for tunability of the labeled peaks. As shown in Figure 2b, deuterium labeling of PE 32:1 is readily observed for a broad range of D<sub>2</sub>O concentrations. Adjusting the D<sub>2</sub>O concentration shifts the position of the Gaussian peak distribution and the average molecular weight of the lipid. The linear correlation between D<sub>2</sub>O concentration and average molecular weight (Figure 2c) suggests no detrimental effect to *E. coli* at least up to 35% D<sub>2</sub>O concentration. At 5% D<sub>2</sub>O, some of the labeled and unlabeled peaks (e.g., natural <sup>13</sup>C<sub>1</sub>-PE 32:1 versus in vivo-labeled <sup>2</sup>H<sub>1</sub>-PE 32:1) are nearly entirely overlapped due to the insufficient mass resolution and difficult to differentiate. At 35% D<sub>2</sub>O, in contrast, the labeled peaks are clearly distinguishable from old lipids but widely distributed. For a complete separation and high deuterium labeling, 20% D<sub>2</sub>O was chosen for subsequent experiments, but any of 10–35% D<sub>2</sub>O could be used. The capability of shifting these labeled peak distributions to higher or lower  $m/z$  provides a means to avoid overlapping background peaks or other lipids. This would be especially useful when applying this method to lower resolution instruments, such as the MALDI Biotyper, to detect labeling from overlapping interferences. Although the washing alleviates signal suppression issues, there are still background interferences in the current work (e.g., labeled with “\*” in Figure 2a). This is not a concern with the current instrumentation as we have sufficient mass resolution to resolve lipids and contamination peaks. However, they can be further removed with additional washing or different workup process for MALDI Biotyper if necessary (data not shown).

In addition to PE 32:1, the most abundant membrane lipid, efficient deuterium labeling is detected for all other lipids (Figure S1). The labeled peaks are well-separated from the unlabeled peak region ( $M \sim M+2$ ). The number of possible deuterium labeling can be calculated as the total number of carbon-bound hydrogens multiplied by D<sub>2</sub>O concentration. For example, PE 32:1 has 69 C-bound hydrogens and 20% of them, 13.8, could be labeled by deuterium. However, the kinetic isotope effect makes in vivo deuterium labeling less efficient than that of hydrogen, mainly in the biosynthesis of nicotinamide adenine dinucleotide phosphate (NADPH) and fatty acids. The efficiency of deuterium labeling can be calculated as the average number of labeled deuterium out of the theoretical number of deuterium labeling. For example, the average of  $\sim 7.6$  D-labeling in PE 32:1 for newly synthesized lipids corresponds to  $\sim 55\%$  fractional abundance of deuterium ( $F_{D\text{-label}}$ ).

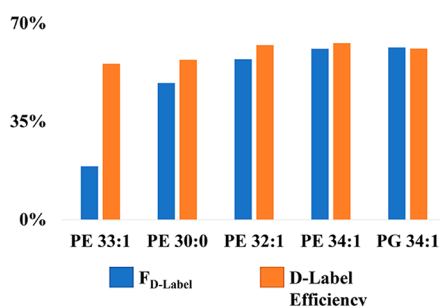
Most mass spectrometers do not have sufficient mass resolution to resolve deuterium versus the <sup>13</sup>C-isotope in the lipid mass range, including the current work. Alternatively, the  $F_{D\text{-label}}$  can be calculated from the average molecular weight (eq 1):

$$F_{D\text{-label}} = \frac{(MW_{D_2O} - MW_{H_2O}) / (m_D - m_H)}{(\text{number of } H_{C\text{-bound}}) \times (D_2O \text{ conc.})} \quad (1)$$

where  $MW_{D_2O}$  and  $MW_{H_2O}$  represent the measured average molecular weight of the lipid species in D<sub>2</sub>O versus H<sub>2</sub>O medium, respectively, and  $m_D - m_H$  represents the mass

difference between deuterium and hydrogen atom mass, 1.006277 Da. Equation 1 is convenient because it is applicable even when there is no clear separation between new and old lipids (e.g., 5% D<sub>2</sub>O in Figure 2b). Additionally, it corrects the contribution from the natural isotopes, including D-labeling of <sup>13</sup>C<sub>1</sub> lipids. However, it is distinguished from D-labeling efficiency in that it accounts for the deuterium abundance out of the total lipids including both old and new lipids.

As shown in Figure 3, the fractional abundance of deuterium varies for different lipid species. PE 30:0, PE 32:1, and PE 34:1



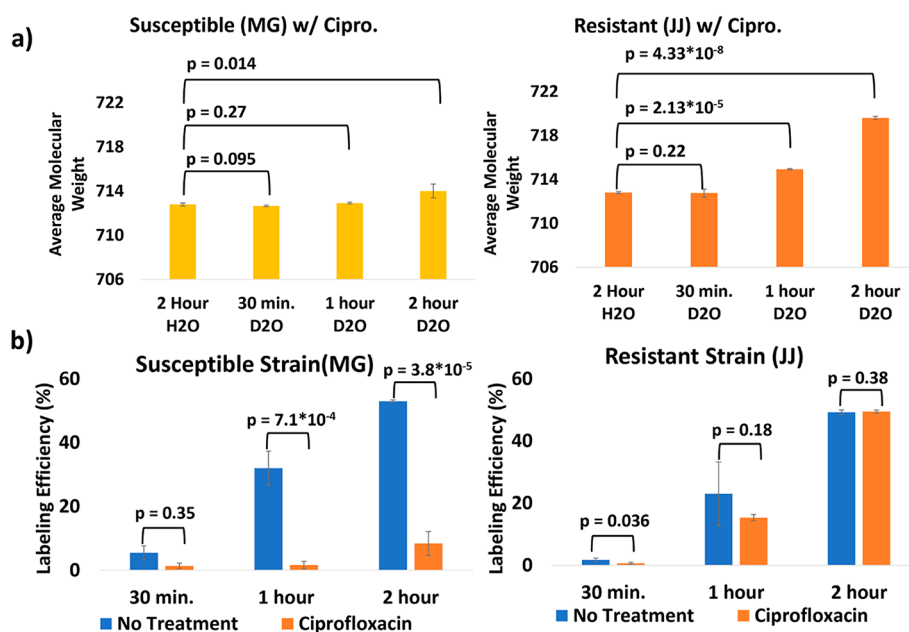
**Figure 3.** Fractional abundance of deuterium calculated for five common membrane lipids in JJ (resistant) strain in 20% D<sub>2</sub>O after 2 h. The D-labeling efficiency is calculated from only new lipids ( $M + 3 - M + 13$ ).

have  $F_{D-label}$  of 45–60% with the  $M + 7$  and  $M + 8$  peaks  $\sim 3.5$ – $5\times$  higher than the monoisotopic peak (Figure S1 and Figure 2a). PG 34:1 has the highest  $F_{D-label}$  of  $\sim 61\%$  with the  $M + 8$  peak roughly  $14\times$  higher than the monoisotopic peak. In contrast, PE 33:1 still has a significant amount of old lipids ( $M \sim M + 2$ ) compared to new lipids with only  $\sim 19\%$  of  $F_{D-label}$ . However, the D-labeling efficiency calculated from the new lipids alone (Figure 3, D-label efficiency, i.e., from  $M + 3$  through  $M + 13$ ) is  $\sim 50$ – $60\%$  and still comparable to that of other lipids (50–60%), suggesting it is due to the lipid

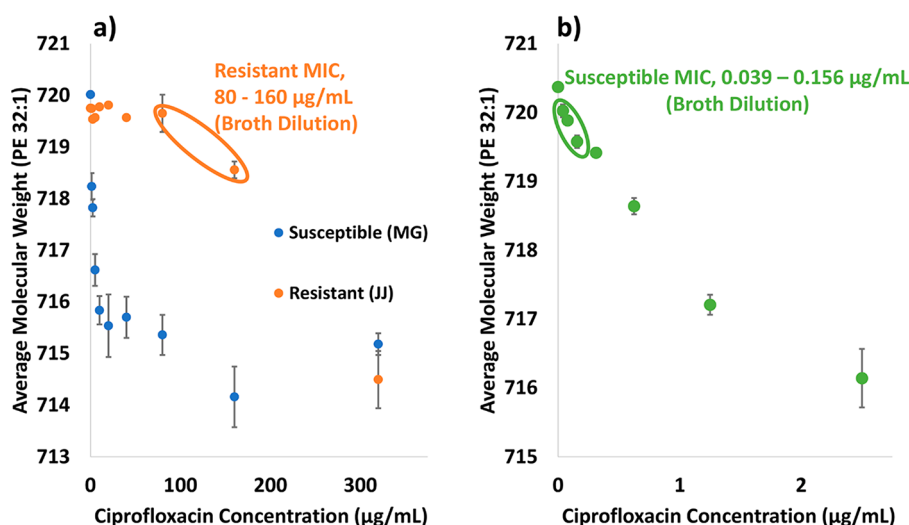
composition change in newly synthesized cell membranes. It is well-known that lipid composition changes depend on the phase of bacterial growth.<sup>28</sup> An additional advantage of this method is that lipids are not unique to particular strains of bacteria, suggesting this method could be universally applicable even for an unknown bacteria.

**Antibiotic Treatment and Minimum Inhibitory Concentration.** The next step is testing the membrane lipid labeling response to antibiotic treatment. Based on previous experiments, PE 32:1 at 20% D<sub>2</sub>O was used for the rest of the experiments. PE 32:1 was chosen for tracking bacterial growth as it was the most abundant lipid in the spectrum. Figure 4a shows the average molecular weight of PE 32:1 after being cultured in 20% D<sub>2</sub>O, or H<sub>2</sub>O for the control, at multiple time points with 10  $\mu\text{g}/\text{mL}$  ciprofloxacin. The D<sub>2</sub>O culture for 30 min is almost indistinguishable from the 2 h H<sub>2</sub>O culture in both strains. Although the labeled peaks are present in the 30 min culture, they are in very low abundance compared to the unlabeled monoisotopic peak. It is mainly attributed to the slow growth in the lag phase. A contrast is observed in the 1 and 2 h cultures, as the average molecular weight increases significantly for the resistant strain (JJ) ( $p$ -value of  $2.13 \times 10^{-5}$  and  $4.33 \times 10^{-8}$ , respectively) but stays relatively consistent for the susceptible strain (MG) ( $p$ -value of 0.27 and 0.014, respectively). This is expected as an effective antibiotic will halt the growth and replication of susceptible bacterial cells, stopping the uptake and incorporation of deuterium in lipid cell membrane biosynthesis. Figure 4b shows the labeling efficiency for each time point with and without treatment. As expected, the discrepancy in labeling is statistically different for the susceptible strain with the  $p$ -value of  $7.1 \times 10^{-4}$  and  $3.8 \times 10^{-5}$  in 1 and 2 h culture, respectively, whereas the treated resistant strain is not statistically different compared to the untreated one.

Finally, an MIC study was performed by MALDI-MS of on-target microdroplet culture with in vivo deuterium labeling.



**Figure 4.** (a) Average molecular weight of PE 32:1 at different time points for MG (susceptible) and JJ (resistant) strains of *E. coli* cultured in 20% D<sub>2</sub>O with 10  $\mu\text{g}/\text{mL}$  ciprofloxacin at three different time points. (b) Labeling efficiency for the same cultures comparing treated versus untreated for susceptible and resistant strains. Error bars are standard deviation from  $n = 3$ .

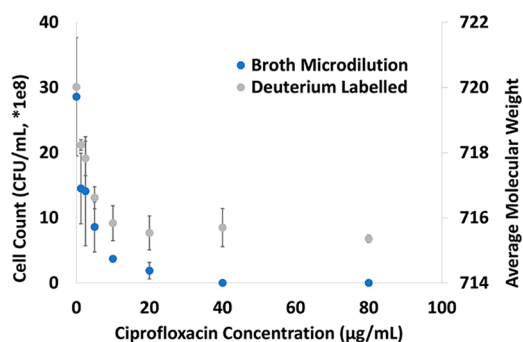


**Figure 5.** (a) Average molecular weight of PE 32:1 for JJ (resistant) and MG (susceptible) after 2 h culture with 20% D<sub>2</sub>O in a range of ciprofloxacin concentrations. (b) Average molecular weight of PE 32:1 for MG after 2 h culture with 20% D<sub>2</sub>O in a narrower range of ciprofloxacin concentrations. Each point was collected in triplicate. Circled points correspond to the traditionally obtained MIC.

Figure 5a shows the average molecular weight of PE 32:1 after a 2 h culture with 20% D<sub>2</sub>O for a range of ciprofloxacin concentrations. The isotope distributions are extracted from the mass spectral data are also shown in Figure S2. The molecular weight of the lipid remains consistent for the resistant strain up to an extreme antibiotic concentration, 80 µg/mL, but at higher concentrations, bacteria cannot process the amount of antibiotic present and are inhibited, resulting in a decrease in the molecular weight. On the other hand, the susceptible strain is inhibited at the first antibiotic concentration as a decrease in labeling is immediately observed. The molecular weight decreases to a plateau across a broad range of concentrations (10 to 320 µg/mL). This is expected as the lowest concentration (1.25 µg/mL) is still far above previously recorded MICs for MG1655 obtained from broth dilution, 0.078 µg/mL, so bacterial growth is strongly inhibited.<sup>30</sup> A similar labeling trend is observed for other lipids, as shown in Figure S3, and differences in the labeling behavior for PE 33:1 can be attributed to slower turnover rate (Figure 3). Additional experiments were performed with a lower range of antibiotic concentrations to determine the MIC of the susceptible MG1655 strain (Figure 5b). A decrease in labeling is observed even for the smallest drug concentration, 0.039 µg/mL, confirming that ciprofloxacin effectively inhibits the strain. Additionally, this highlights that the method is sensitive enough to detect slight decreases in labeling (<0.5 Da) at low antibiotic concentrations. For both resistant and susceptible strains, the average molecular weight at the very high concentration does not decrease to that of control (714.5–715.2 versus 712.8 Da). This is because some newly labeled lipids are produced before cell death.

These data on their own are useful in determining resistant and susceptible strains as the decrease in labeling indicates antibiotic efficacy. However, a comparison to a traditional MIC measurement is necessary to quantitatively validate the deuterium labeling MIC for its potential clinical use. To accomplish this, a broth dilution experiment was used to determine the concentration of ciprofloxacin, which inhibits the resistant and susceptible strains. This method takes about 3 days total and requires manual counting of the colonies on the agar plate. The approximate concentration where the number

of colonies decreases compared to the untreated control indicates the MIC. The MIC determined by this broth dilution method is indicated in Figure 5 to compare with the deuterium labeling MIC. The traditional MIC for the resistant strain is between 80 and 160 µg/mL ciprofloxacin, which corresponds to the first point in the deuterium labeling MIC, where the labeling starts to decrease. The susceptible strain has a much lower MIC range between 0.039 and 0.156 µg/mL but also corresponds to the decrease in the deuterium labeling. In addition, the overall cell count from the broth dilution at each concentration also corresponds well to the deuterium labeling method (Figure 6). These results indicate that this method can not only differentiate between resistant and susceptible strains but also be directly related to the MIC value for each strain.



**Figure 6.** Comparison of the average molecular weight of PE 32:1 for MG (susceptible) from the deuterium labeling experiment versus the cell count obtained from a traditional broth dilution experiment. The decrease in cell count and the decrease in labeling correlate well over a range of ciprofloxacin concentrations.

## CONCLUSION

In this work, we developed an on-target microdroplet culturing method with D<sub>2</sub>O to label bacterial membrane lipids and track bacterial growth. When being cultured with a range of antibiotics, the resistance of a strain can be determined by the  $F_{D\text{-label}}$  or average molecular weight of common membrane lipids, such as PE 32:1 in *E. coli*. In addition, the MIC can be

approximated by the first antibiotic concentration at which the labeling efficiency begins to decrease, which gives comparable MIC values obtained by a traditional broth dilution method. Typical MIC experiments have slow TATs, while the deuterium labeling experiment takes only 1 or 2 h to see the clear resistance. Although this work is a proof of concept which requires an overnight stock culture, we hypothesize that further development will allow for direct labeling analysis of isolated clinical strains. Furthermore, MALDI-MS data acquisition can be very fast, as little as a few minutes per plate, which means that multiple strains, antibiotics, or antibiotic concentrations can be cultured and analyzed in a single high-throughput experiment. The material demands of the experiment are also much lower than those of traditional methods as only a small volume of bacteria culture and antibiotic/D<sub>2</sub>O are necessary for each culture. Overall, the deuterium labeling method proposed here provides a high-throughput, low material method to determine MIC and, more broadly, the resistance or susceptibility of a particular strain of bacteria. This approach should be applicable regardless of antibiotic, bacterial strain, or resistance mechanism.

One of the next steps for this work will be further studying other bacteria and antibiotic combinations. *E. coli* are fast-growing, Gram-negative bacteria. Other bacteria may take longer to culture and produce enough labeled signals; however, their growth should be detected faster than other traditional growth methods. Different species and strains will have different lipid distributions, and background peaks may overlap the most abundant labeled lipids. The deuterium concentration can be adjusted to shift the lipid distributions to avoid contamination. These steps will require some optimization, but the general workflow will remain the same and the adaptation to new bacteria and antibiotics could be easily made. Finally, applying the method to more popular mass spectrometers in clinical laboratories, such as a Bruker Biotyper, will be a significant milestone to bring it to broader applications. As the Biotyper has a much lower resolution than the current Orbitrap instrumentation, optimization will be necessary, but the labeled peaks should be detectable, especially when compared to the unlabeled spectrum. Adapting to the Biotyper would also allow for simultaneous identification of the species or strain present while also acquiring MIC information using the deuterium labeling method.

## ■ ASSOCIATED CONTENT

### SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jasms.2c00056>.

Distribution of deuterium-labeled peaks for four additional lipids in 20% D<sub>2</sub>O, distribution of deuterium-labeled PE 32:1 peaks for JJ and MG in a range of cipro concentrations, average molecular weight of three additional lipids after 2 h labeling in a range of cipro concentrations (PDF)

## ■ AUTHOR INFORMATION

### Corresponding Author

Young Jin Lee – Department of Chemistry, Iowa State University, Ames, Iowa 50011, United States; [orcid.org/0000-0002-2533-5371](https://orcid.org/0000-0002-2533-5371); Email: [yjlee@iastate.edu](mailto:yjlee@iastate.edu)

## Authors

Evan A. Larson – Department of Chemistry, Iowa State University, Ames, Iowa 50011, United States  
Josiah J. Rensner – Department of Chemistry, Iowa State University, Ames, Iowa 50011, United States  
Kristina R. Larsen – Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, Iowa 50011, United States  
Bryan Bellaire – Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, Iowa 50011, United States; [orcid.org/0000-0002-4034-6482](https://orcid.org/0000-0002-4034-6482)

Complete contact information is available at: <https://pubs.acs.org/10.1021/jasms.2c00056>

## Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

This work is supported by a seed fund from Antimicrobial Resistance Consortium, Iowa State University.

## ■ REFERENCES

- (1) Pendleton, J. N.; Gorman, S. P.; Gilmore, B. F. Clinical Relevance of the ESKAPE Pathogens. *Expert Rev. Anti-Infect. Ther.* **2013**, *11* (3), 297–308.
- (2) Mulani, M. S.; Kamble, E. E.; Kumkar, S. N.; Tawre, M. S.; Pardesi, K. R. Emerging Strategies to Combat ESKAPE Pathogens in the Era of Antimicrobial Resistance: A Review. *Front. Microbiol.* **2019**, *10*, 539.
- (3) Leonard, H.; Colodner, R.; Halachmi, S.; Segal, E. Recent Advances in the Race to Design a Rapid Diagnostic Test for Antimicrobial Resistance. *ACS Sens.* **2018**, *3* (11), 2202–2217.
- (4) Reynoso, E. C.; Laschi, S.; Palchetti, I.; Torres, E. Advances in Antimicrobial Resistance Monitoring Using Sensors and Biosensors: A Review. *Chemosensors* **2021**, *9* (8), 232.
- (5) Chen, C.; Hong, W. Recent Development of Rapid Antimicrobial Susceptibility Testing Methods through Metabolic Profiling of Bacteria. *Antibiotics* **2021**, *10* (3), 311.
- (6) van Belkum, A.; Burnham, C.-A. D.; Rossen, J. W. A.; Mallard, F.; Rochas, O.; Dunne, W. M. Innovative and Rapid Antimicrobial Susceptibility Testing Systems. *Nat. Rev. Microbiol.* **2020**, *18* (5), 299–311.
- (7) Smith, K. P.; Kirby, J. E. Rapid Susceptibility Testing Methods. *Clin. Lab. Med.* **2019**, *39* (3), 333–344.
- (8) Idelevich, E. A.; Becker, K. How to Accelerate Antimicrobial Susceptibility Testing. *Clin. Microbiol. Infect.* **2019**, *25* (11), 1347–1355.
- (9) Zhu, W.; Sieradzki, K.; Albrecht, V.; McAllister, S.; Lin, W.; Stuchlik, O.; Limbago, B.; Pohl, J.; Kamile Rasheed, J. Evaluation of the Biotyper MALDI-TOF MS System for Identification of Staphylococcus Species. *J. Microbiol. Methods* **2015**, *117*, 14–17.
- (10) Faron, M. L.; Buchan, B. W.; Hyke, J.; Madisen, N.; Lillie, J. L.; Granato, P. A.; Wilson, D. A.; Procop, G. W.; Novak-Weekley, S.; Marlowe, E.; Cumpio, J.; Griego-Fullbright, C.; Kindig, S.; Timm, K.; Young, S.; Ledebner, N. A. Multicenter Evaluation of the Bruker MALDI Biotyper CA System for the Identification of Clinical Aerobic Gram-Negative Bacterial Isolates. *PLoS One* **2015**, *10* (11), No. e0141350.
- (11) Lee, W.; Kim, M.; Yong, D.; Jeong, S. H.; Lee, K.; Chong, Y. Evaluation of VITEK Mass Spectrometry (MS), a Matrix-Assisted Laser Desorption Ionization Time-of-Flight MS System for Identification of Anaerobic Bacteria. *Ann. Lab. Med.* **2015**, *35* (1), 69–75.
- (12) Idelevich, E. A.; Sparbier, K.; Kostrzewa, M.; Becker, K. Rapid Detection of Antibiotic Resistance by MALDI-TOF Mass Spectrometry

etry Using a Novel Direct-on-Target Microdroplet Growth Assay. *Clin. Microbiol. Infect.* **2018**, *24* (7), 738–743.

(13) Idelevich, E. A.; Storck, L. M.; Sparbier, K.; Drews, O.; Kostrzewa, M.; Becker, K. Rapid Direct Susceptibility Testing from Positive Blood Cultures by the Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry-Based Direct-on-Target Microdroplet Growth Assay. *J. Clin. Microbiol.* **2018**, *56* (10), No. e00913-18.

(14) Correa-Martínez, C. L.; Idelevich, E. A.; Sparbier, K.; Kostrzewa, M.; Becker, K. Rapid Detection of Extended-Spectrum  $\beta$ -Lactamases (ESBL) and AmpC  $\beta$ -Lactamases in Enterobacterales: Development of a Screening Panel Using the MALDI-TOF MS-Based Direct-on-Target Microdroplet Growth Assay. *Front. Microbiol.* **2019**, *10*, 13.

(15) Nix, I. D.; Idelevich, E. A.; Storck, L. M.; Sparbier, K.; Drews, O.; Kostrzewa, M.; Becker, K. Detection of Methicillin Resistance in *Staphylococcus aureus* from Agar Cultures and Directly From Positive Blood Cultures Using MALDI-TOF Mass Spectrometry-Based Direct-on-Target Microdroplet Growth Assay. *Front. Microbiol.* **2020**, *11*. DOI: 10.3389/fmicb.2020.00232

(16) Sorensen, M.; Chandler, C. E.; Gardner, F. M.; Ramadan, S.; Khot, P. D.; Leung, L. M.; Farrance, C. E.; Goodlett, D. R.; Ernst, R. K.; Nilsson, E. Rapid Microbial Identification and Colistin Resistance Detection via MALDI-TOF MS Using a Novel on-Target Extraction of Membrane Lipids. *Sci. Rep.* **2020**, *10* (1), 21536.

(17) Leung, L. M.; McElheny, C. L.; Gardner, F. M.; Chandler, C. E.; Bowler, S. L.; Mettus, R. T.; Spychala, C. N.; Fowler, E. L.; Opene, B. N. A.; Myers, R. A.; Goodlett, D. R.; Doi, Y.; Ernst, R. K. A Prospective Study of *Acinetobacter baumannii* Complex Isolates and Colistin Susceptibility Monitoring by Mass Spectrometry of Microbial Membrane Glycolipids. *J. Clin. Microbiol.* **2018**, *57*, e01100-18.

(18) Fondrie, W. E.; Liang, T.; Oyler, B. L.; Leung, L. M.; Ernst, R. K.; Strickland, D. K.; Goodlett, D. R. Pathogen Identification Direct From Polymicrobial Specimens Using Membrane Glycolipids. *Sci. Rep.* **2018**, *8* (1), 15857.

(19) Ting, Y. S.; Shaffer, S. A.; Jones, J. W.; Ng, W. V.; Ernst, R. K.; Goodlett, D. R. Automated Lipid A Structure Assignment from Hierarchical Tandem Mass Spectrometry Data. *J. Am. Soc. Mass Spectrom.* **2011**, *22* (5), 856–866.

(20) Liang, T.; Leung, L. M.; Opene, B.; Fondrie, W. E.; Lee, Y. I.; Chandler, C. E.; Yoon, S. H.; Doi, Y.; Ernst, R. K.; Goodlett, D. R. Rapid Microbial Identification and Antibiotic Resistance Detection by Mass Spectrometric Analysis of Membrane Lipids. *Anal. Chem.* **2019**, *91* (2), 1286–1294.

(21) Leung, L. M.; Fondrie, W. E.; Doi, Y.; Johnson, J. K.; Strickland, D. K.; Ernst, R. K.; Goodlett, D. R. Identification of the ESKAPE Pathogens by Mass Spectrometric Analysis of Microbial Membrane Glycolipids. *Sci. Rep.* **2017**, *7* (1), 6403.

(22) Zhang, R.; Qin, Q.; Liu, B.; Qiao, L. TiO<sub>2</sub>-Assisted Laser Desorption/Ionization Mass Spectrometry for Rapid Profiling of Candidate Metabolite Biomarkers from Antimicrobial-Resistant Bacteria. *Anal. Chem.* **2018**, *90* (6), 3863–3870.

(23) Burckhardt, I.; Zimmermann, S. Using Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry To Detect Carbapenem Resistance within 1 to 2.5 h. *J. Clin. Microbiol.* **2011**, *49* (9), 3321–3324.

(24) Oviño, M.; Sparbier, K.; Barba, M. J.; Kostrzewa, M.; Bou, G. Universal Protocol for the Rapid Automated Detection of Carbapenem-Resistant Gram-Negative Bacilli Directly from Blood Cultures by Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF/MS). *Int. J. Antimicrob. Agents.* **2016**, *48* (6), 655–660.

(25) Berry, D.; Mader, E.; Lee, T. K.; Woebken, D.; Wang, Y.; Zhu, D.; Palatinszky, M.; Schintlmeister, A.; Schmid, M. C.; Hanson, B. T.; Shterzer, N.; Mizrahi, I.; Rauch, I.; Decker, T.; Bocklitz, T.; Popp, J.; Gibson, C. M.; Fowler, P. W.; Huang, W. E.; Wagner, M. Tracking Heavy Water (D<sub>2</sub>O) Incorporation for Identifying and Sorting Active Microbial Cells. *Proc. Natl. Acad. Sci. U.S.A.* **2015**, *112* (2), E194–E203.

(26) Tao, Y.; Wang, Y.; Huang, S.; Zhu, P.; Huang, W. E.; Ling, J.; Xu, J. Metabolic-Activity-Based Assessment of Antimicrobial Effects by D<sub>2</sub>O-Labeled Single-Cell Raman Microspectroscopy. *Anal. Chem.* **2017**, *89* (7), 4108–4115.

(27) Kopf, S. H.; Sessions, A. L.; Cowley, E. S.; Reyes, C.; Van Sambeek, L.; Hu, Y.; Orphan, V. J.; Kato, R.; Newman, D. K. Trace Incorporation of Heavy Water Reveals Slow and Heterogeneous Pathogen Growth Rates in Cystic Fibrosis Sputum. *Proc. Natl. Acad. Sci. U. S. A.* **2016**, *113* (2), E110–116.

(28) Neubauer, C.; Sessions, A. L.; Booth, I. R.; Bowen, B. P.; Kopf, S. H.; Newman, D. K.; Dalleska, N. F. Towards Measuring Growth Rates of Pathogens during Infections by D<sub>2</sub> O-labeling Lipidomics. *Rapid Commun. Mass Spectrom.* **2018**, *32* (24), 2129–2140.

(29) Lehmann, W. D. A Timeline of Stable Isotopes and Mass Spectrometry in the Life Sciences: A TIMELINE OF STABLE ISOTOPES AND MASS SPECTROMETRY. *Mass. Spec. Rev.* **2017**, *36* (1), 58–85.

(30) Ching, C.; Zaman, M. H. Development and Selection of Low-Level Multi-Drug Resistance over an Extended Range of Sub-Inhibitory Ciprofloxacin Concentrations in *Escherichia Coli*. *Sci. Rep.* **2020**, *10* (1), 8754.