Ambient generation of fatty acid methyl ester ions from bacterial whole cells by direct analysis in real time (DART) mass spectrometry†

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Direct analysis in real time (DART) is implemented on a time-of-flight (TOF) mass spectrometer, and used for the generation of fatty acid methyl esters (FAMEs) ions from whole bacterial cells.

Mass spectrometry (MS) is a well established tool for addressing biological problems. Through the work of many research groups, and in particular of Fenselau's, it has been shown that the application of MS to biomarker discovery for microbial identification offers a powerful complement to traditional microbiological approaches.[1] In the last decade, a wide variety of desorption/ionization methods for MS has been developed and subsequently applied to microbial identification problems.[2] Recently, several new high throughput "ambient" ionization methods that operate in open air have been reported.[3] Two of the most studied ambient ionization methods have been desorption electrospray ionization (DESI)[4] and direct analysis in real time (DART).[5] (References to other ambient ionization methods can be found in the ESI.†)

Takáts *et al.* were the first to recognize the potential of these ambient ionization methods for microorganism identification, showing DESI mass spectra of freshly harvested, untreated *Escherichia coli* and *Pseudomonas aeruginosa* samples deposited on PTFE.[6] However, the potential of other ambient ionization methods, such as DART, for generating ions from microbial samples has not been yet reported. This communication describes our first results on the generation of fatty acid methyl ester ions from whole bacterial cell suspensions by DART, and their identification by accurate-mass orthogonal TOF MS.

DART is conducted in open air, allowing for the rapid, non-contact analysis of solid, liquid, and gaseous materials without sample preparation.[5] The DART process begins with a He stream that supports a point-to-plane corona discharge. A series of processes within this discharge (electron-impact, ion-electron recombination) produce metastable He atoms (He* $^3S_1$, 19.8 eV), which are carried downstream by the gas. Immediately after the region where the discharge takes place, the gas stream is heated to temperatures that can be varied from 150 to 450 °C. Although the DART ionization mechanisms are not yet fully understood, it has been proposed that, upon exiting the ion source, He* atoms induce Penning ionization of atmospheric water, generating protonated water clusters.[5] Gaseous analytes vaporized from the solid or liquid sample react with these clusters, forming protonated adduct ions. A more detailed discussion of DART ionization can be found elsewhere.[5,7,8]

As recently described by Fox,[9] one of the "gold standard" methods routinely used in the field of clinical bacterial identification is based on the determination of microbial fatty acid methyl ester (FAME) composition after culture, which forms the basis of the commercial Sherlock® microbial identification system (MIDI Inc., Newark, Delaware, US). FAME composition analysis starts with the saponification of the lipidic material in bacterial cells, followed by fatty acid methylation, and gas chromatographic analysis with flame ionization or mass spectrometric detection. Sample preparation for this method takes several hours, and each chromatographic run can take from 20 to 30 min.[9]

Alternative approaches to FAME composition analysis that avoid the chromatographic separation step have also been reported. For example, Xu *et al.* described a method that relies on *in situ* lipid hydrolysis and methylation of bacterial samples to generate FAMEs, followed by chemical ionization (CI) MS.[10] As CI occurs by proton transfer, it has the advantage of reducing the amount of internal energy deposited on the generated ions, thus significantly reducing FAME ion fragmentation. For most analytes, DART ionization has also been found to occur by proton transfer, as in CI. In addition, we have observed that DART sensitivity is particularly good for volatile or semi-volatile molecules, such as FAMEs. Due to these concurrent features, it seems logical to investigate the feasibility of producing FAME ions by DART. The successful demonstration of the generation of FAME ions from bacterial whole cells *via* DART constitutes the first step that could lead, in the future, to the successful development of new approaches for high throughput microbial identification in a variety of biological, foodstuff, and water samples in the open air, with minimum sample preparation.

FAMEs were generated from whole cell bacterial suspensions (*Streptococcus pyogenes* ATCC 700294, 10E7 cell μL^{-1}; *E. coli* ATCC 25922, 10E7 cell μL^{-1}; γ-irradiated *Coxiella burnetii*, 10E8 cell μL^{-1}). *S. pyogenes* and *E. coli* were cultured by inoculation in tubes containing 30 ml of Todd Hewitt broth (THB). After incubation at 37 °C for 18–24 h, cells were washed three times with 10 ml of TRIS-sucrose buffer (0.01 M TRIS,

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0.025 M sucrose, pH 7.0) by centrifugation under refrigeration (4–10 °C). Cells were then suspended in 500 μl of ultrapure water, and kept at −80 °C. All *C. burnetii* strains were cultured in identical conditions as described previously.[11] Aqueous bacterial suspensions were diluted 1 : 1 (v/v) with a 0.27 M solution of tetramethylammonium hydroxide (TMAH) (+99%, Aldrich, Milwaukee, WI) to produce thermal hydrolysis and methylation of bacterial lipids. The DART ion source was interfaced to a JMS-100TLC (AccuTOF™) orthogonal time-of-flight mass spectrometer (JEOL, USA, Peabody, MA) operated in positive-ion mode (Fig. S1, ESI†). Helium gas (7.0 L min^{-1}) was introduced into the DART corona discharge chamber where a needle electrode was held at −3000 V. This discharge is physically separated from the open-air ionization region where the sample is placed by two small cylindrical chambers, each ending in a DC-biased electrode. The first electrode was held at 300 V and the exit electrode at 150 V. The gas flow and electrode potentials have a broad operating maximum and many combinations of settings presented similar sensitivities. The DART ion source was positioned in front of the mass spectrometer inlet orifice. The inlet orifice voltage was 54 V *vs*. ground. The distance between the DART ion source exit, and the spectrometer inlet was 20 mm. A sliding arm was used to assure reproducible sample positioning within the DART ionizing stream. An alligator clip secured to this arm was used to hold a 1.5 o.d. × 90 mm long glass capillary tube in the vertical position. A 4 μL aliquot of the whole bacterial cell suspension mixed with TMAH was deposited directly with a micropipette to the bottom of the capillary tube. The capillary was positioned so that, after sliding the sample holder arm, the bottom of the tube came in contact with the DART He stream directly in front of the mass spectrometer inlet orifice. Clean, unused, capillary tubes were employed each time. The DART ion source was heated following a rapid temperature gradient. The initial temperature was set at 150 °C, and data acquisition was started at t = 0 min. Data were acquired for 1 min for background correction purposes. At t = 1 min the sliding sample holder was quickly shifted, placing the capillary (with the sample on its surface) in front of the spectrometer inlet. The temperature controller was simultaneously switched to ramp to 500 °C in 3 min. Spectral data were acquired until the total ion signal intensity completely decreased to background levels at t = 7 min. The sample was then removed from the ionization region. At t = 8 min a separate capillary tube loaded with neat poly(ethylene glycol) (PEG, average molecular weight 600) was briefly (1–2 s) introduced into the helium stream to obtain a reference mass spectrum to perform TOF accurate mass measurements. Data acquisition was stopped at t = 9 min. The fatty acids investigated ranged from C8:1 (Cn:x, n = number of carbons and x = number of unsaturations) to C24:0. Spectra were acquired every 250 ms in the 150 to 600 u mass range.

Fig. 1(a) and (b) show the *E. coli* and *S. pyogenes* reconstructed ion "chronograms" obtained by DART-TOF MS. It can be seen that when the DART temperature is ramped, discrimination in the temporal domain occurs. Identical FAMEs show different time evolution profiles for different bacterial samples. These differences are probably caused by differences in the hydrolysis and desorption rates. In both cases the C8:0 FAME is desorbed earlier in the temperature gradient, but with a different peak shape for each sample. With this approach, the analysis time was 9 min, however the relevant FAME data is produced in the first 3–4 min,

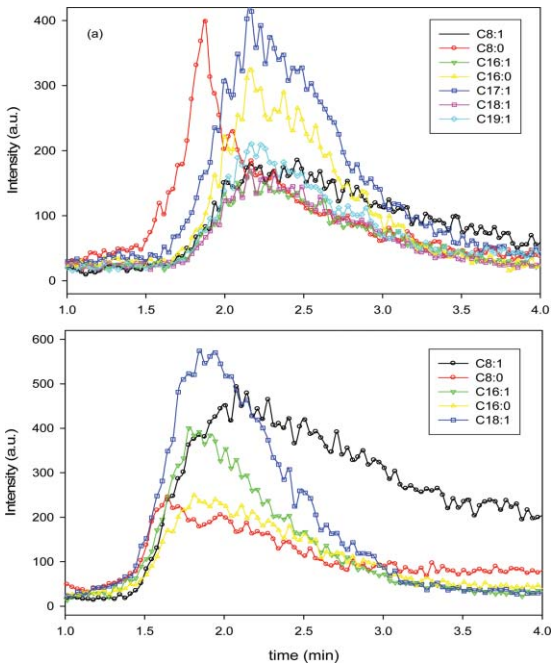

Fig. 1 (a) Reconstructed ion chronograms for *E. coli*. (b) Reconstructed ion chronograms for *S. pyogenes*. Only FAME ions which had a relative intensity greater than 5% are shown.

indicating that the procedure could be further optimized. Table S1, ESI,† shows the types of ions identified by accurate mass measurements, their theoretical *m*/*z*, and their relative abundances for *E. coli*, and *S. pyogenes* samples. Spectral data for *C. burnetii*, which we discuss later, is also presented. No attempt was made to identify FAME peaks with *m*/*z* lower than 150 or above 400 because bacterial FAME MS analysis is generally restricted to this *m*/*z* range. From these profiles, mass spectra were then obtained by averaging multiple scans within a 10 s window at the maximum of the total ion chronogram. Fig. 2(a) illustrates the DART-TOF mass spectrum for *E. coli*. For this bacterium, it has been reported that C14:0 (myristic acid), C16:0 (palmitic acid), C16:1 (palmitoleic acid), C17:1/cycloC17:0, C18:1 (oleic acid), and C19:1/cycloC19:0 account for over 98% of total fatty acid components.[10] The experimental spectrum in Fig. 2(a) shows that these fatty acids were all detectable as protonated methyl esters, with C17:1/cycloC17:0 being the most abundant. The protonated fatty acids methyl esters of C16:1, C16:0, and C18:1 dominated the spectrum for *S. pyogenes*, with C18:1 having the highest intensity (Fig. 2(b)). C9:0, C10:0, C11:0, C12:0, C14:0, C15:0, C17:1/cycloC17:0, and C19:1/cycloC19:0 were found to be present in *E. coli* only, while C11:1 was uniquely detected in *S. pyogenes*. C17:1/cycloC17:0 and C19:1/cycloC19:0 were found in *E. coli* at relatively high abundances but were not detected in the *S. pyogenes* spectrum, in agreement with the membrane characteristics of Gram negative bacteria.[10] Some FAME ions were common to *E. coli*, and *S. pyogenes*, however clear differences existed in the relative abundances of these ions in the respective mass spectra. Differences among samples were thus observed in the spectral, temporal and intensity domains. The FAME spectra obtained by DART-TOF MS were not identical to the ones obtained by CI-based methods.[10] However; these differences are to be expected, as differences in sample growth conditions, or in the ion yields or ion

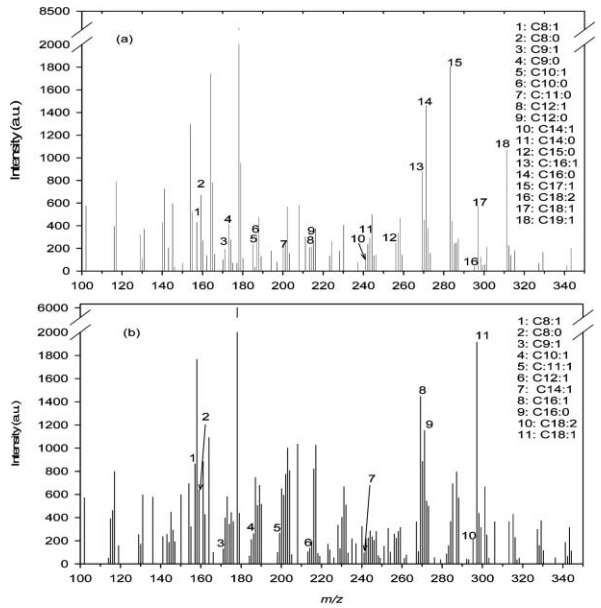

Fig. 2 Positive ion mass spectrum of (a) *E. coli* (Gram negative) and (b) *S. pyogenes* (Gram positive) acquired by direct DART-TOF MS analysis after *in-situ* thermal hydrolysis/methylation of the bacterial fatty acids to generate the corresponding FAMEs.

transmission of the different instruments used could affect the observed FAME ion intensities.[8] Notwithstanding these differences, our findings indicate that it is possible to generate FAME ions from bacterial samples by DART.

In order to further investigate the types of ions observed from different bacterial samples, three strains of *Coxiella burnetii*, were investigated by our DART-TOF MS approach. *C. burnetii* is a highly infective microorganism, causative of the zoonotic disease known as Q-fever. Fig. 3 shows the *C. burnetii* (Nine Mile I strain) FAME mass spectrum obtained by DART-TOF MS. Several FAMEs were identified, with C9:0, C12:0, C15:1, C15:0, C16:0, and C17:0 being the most abundant; and C15:0 alone accounting for over 38% of the total signal intensity. C15:1, C17:0, C19:0, C21:1, C21:0, C24:1 and C24:0 were found to be unique to *C. burnetii* Nine Mile I when compared to *E. coli* and *S. pyogenes* (Table S1, ESI†), exhibiting a distinct mass spectrum (Fig. 3). DART-TOF mass spectra for two other *C. burnetii* strains (Nine Mile II and RSA 514) are shown in the electronic supplementary information section (Fig. S2, ESI†), showing marked spectral differences with the Nine Mile I strain.

In conclusion, our results show that DART-TOF MS can successfully produce fatty acid methyl ester mass spectra from various bacterial samples. The method is fast and operates in open air, opening the possibility of investigating the presence of microorganisms on other types of surfaces. The FAME mass spectra were generated using a simple procedure, which involved co-deposition of intact bacteria and TMAH solution prior to

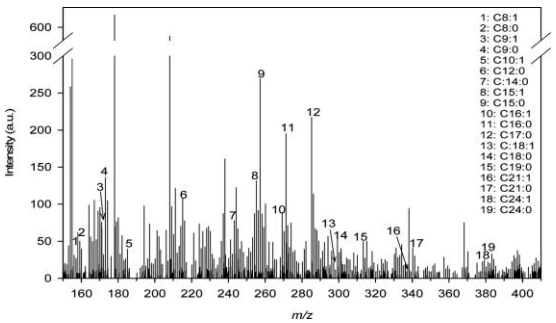

Fig. 3 Positive ion mass spectrum of *C. burnetii* Nine Mile I (Gram negative) acquired by DART-TOF MS.

DART-TOF MS analysis. So far, our mass spectral analysis has been performed by simple visual inspection in one of the analytical dimension at a time (*i.e.* time, *m/z*, or intensity), but more sophisticated multiway data analysis approaches, such as parallel factor analysis (PARAFAC) could also be applied to this type of data.[12] Future research will focus on improving sensitivity, studying bacterial mixtures, and the effect of different ionization and culture conditions on the observed mass spectra.

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