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Direct Analysis of Intact Proteins from *Escherichia coli* Colonies by Liquid Extraction Surface Analysis Mass Spectrometry

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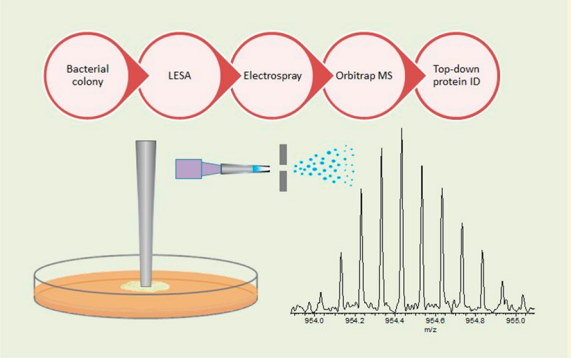
Supporting Information

ABSTRACT: Top-down identification of proteins by liquid extraction surface analysis (LESA) mass spectrometry has previously been reported for tissue sections and dried blood spot samples. Here, we present a modified "contact" LESA method for top-down analysis of proteins directly from living bacterial colonies grown in Petri dishes, without any sample pretreatment. It was possible to identify a number of proteins by use of collision-induced dissociation tandem mass spectrometry followed by searches of the data against an *E. coli* protein database. The proteins identified suggest that the method may provide insight into the bacterial response to environmental conditions. Moreover, the results show that the "contact" LESA approach results in a smaller sampling area than typical LESA, which may have implications for spatial profiling.



It was first demonstrated that mass spectrometry could be applied to the identification and differentiation of species of bacteria over 30 years ago;[1,2] subsequent advances in software and instrumentation now make it a powerful tool for microbiological research.[3,4] MALDI-TOF mass spectrometry has been reported for the identification of bacterial species from isolates of over 1000 species. Identification involves searching mass spectra against spectral libraries of abundant intact proteins which are characteristic of that species.[2,3] It has been suggested that this method will replace traditional identification methods such as Gram stain, colony morphology, and biochemical tests.[3] MALDI MS has also been used to image bacterial colonies with a spatial resolution capable of capturing metabolic output of bacteria, which can be compared between mutant and wildtype strains.[5−7] The identification of metabolites from mass spectral images of bacteria can help in the elucidation of biochemical pathways underlying microbial processes, which are often poorly characterized. Techniques capable of detecting chemical signals in a spatially resolved manner could also aid our understanding of bacterial inter- and intraspecies interactions.[8]

The majority of proteins detected by MALDI methods are small ribosomal proteins (typically <15 kDa) which are ideally suited to and relied upon for the robust identification of bacterial species and subspecies. However, the routine detection of larger bacterial proteins remains elusive. Electrospray ionization (ESI) top-down mass spectrometry methods provide an increased mass range and total number of proteins detected. Top-down analysis[9] involves fragmentation of intact protein ions and overcomes the limitation of information loss suffered by bottom-up analysis[10] of proteolytic peptides. For example, Fenselau and co-workers[11] applied top-down LC MS/MS to the analysis of bacterial lysates from *Erwinia herbicola* and *Enterobacter cloacae*. Fourteen proteins were identified in *E. herbicola*, ten of which were ribosomal. Fifteen proteins were identified in *E. cloacae*, five of which were ribosomal and three were cold shock proteins. More recently, McFarland et al.[12] applied top-down LC MS/MS to the analysis of lysates from *Salmonella enterica* serovars. They identified 73 proteins in the mass range of 4−36 kDa.

The development of ambient mass spectrometry techniques such as desorption electrospray ionization (DESI)[13] and liquid extraction surface analysis (LESA)[14] have enabled direct sampling of bacterial colonies with little or no sample preparation. DESI involves desorbing analytes from a surface by directing a stream of charged electrospray droplets at the surface of interest. The sample inlet of the mass spectrometer is positioned above the surface at the optimum angle to capture analyte ions which have been desorbed. LESA involves using a droplet of solvent into which soluble analytes are extracted. The droplet is dispensed from a pipet tip and held in contact with the sample surface for a specified length of time to form a liquid microjunction. The droplet is then reaspirated and injected through an electrospray capillary into the mass spectrometer. These techniques benefit from the advantages provided by ESI-MS, with the added bonus of direct surface sampling. One

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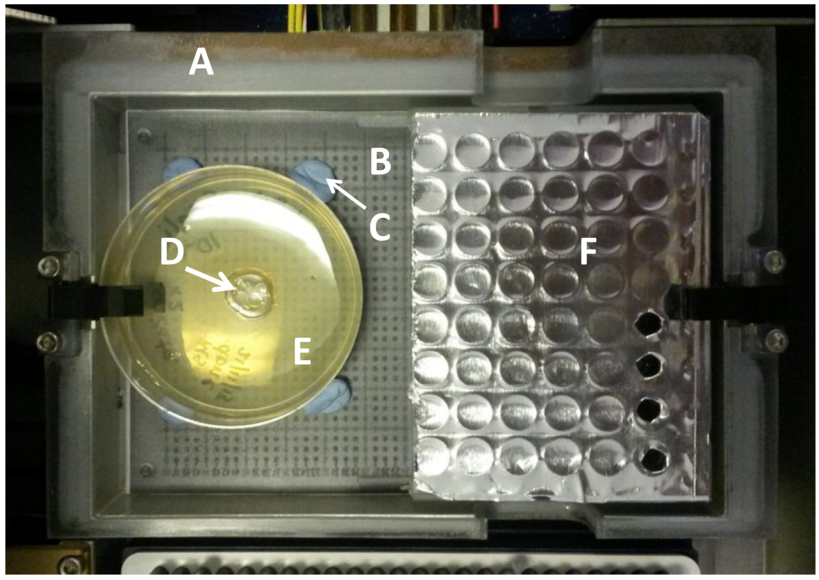

Figure 1. Experimental setup: (A) sample tray of Triversa Nanomate; (B) photocopy of the universal adaptor plate, for *x,y* coordinates; (C) adhesive putty (Blu-tack) to secure Petri dish in position; (D) *E. coli* colony; (E) Petri dish containing agar; (F) half 96-well plate containing extraction/electrospray solvent system.

possible application of these methods is the direct sampling of surfaces contaminated with unknown strains of bacteria, which could have implications for clinical diagnostics, quality control, and environmental monitoring.

Ambient mass spectrometry techniques are ideal for the analysis of bacterial colonies because they can directly sample living colonies growing in Petri dishes.[15] A recent report outlines the spatially resolved detection of glycolipids and metabolites from living bacterial colonies using nano-DESI.[16] This technique was capable of detecting metabolites secreted by bacteria into the surrounding agar without any sample preparation or pretreatment. A further paper detailed the use of nano-DESI to study interactions between neighboring colonies and in mixed-species biofilms,[17] with detection of a wide range of lipids, peptides, and other small molecules reported. A further motivator for the chemical analysis of bacteria is the search for novel therapeutic agents: more than 60% of current pharmaceuticals have been developed from natural products.[8] It has been demonstrated that a thiazolyl peptide antibiotic could be extracted from *Actinobacteria* colonies by LESA and analyzed by nanoelectrospray mass spectrometry.[18] In 2013, the use of an ambient electrospray ionization flow-probe was demonstrated for the metabolic profiling of a range of microorganisms. Diverse classes of natural products have been detected using the flow-probe including small redox compounds, lipids, and peptides.[19] Although a wide variety of small molecules has been detected from bacterial colonies by techniques such as DESI, nano-DESI, LESA, and the continuous flow-probe coupled to mass spectrometry, analysis of intact proteins from bacteria by ambient mass spectrometry has not previously been reported.

LESA-MS has been demonstrated to be capable of extracting intact proteins directly from surfaces without any pretreatment. In 2011, Edwards et al. reported a LESA-MS method for detecting hemoglobin variants from neonatal dried blood spot samples. The mass spectra obtained contained peaks corresponding to the different subunits of hemoglobin, and it was possible to confirm the presence of known variants, including those differing in mass by <1 Da, by MS/MS.[20] It was later shown that LESA MS/MS could be applied for the analysis of unknown hemoglobin variants.[21] Schey et al. reported the liquid extraction of intact proteins from tissue sections. An acetonitrile/water solvent system was used to manually extract proteins from thin tissue sections of mouse brain, mouse kidney, and bovine ocular lens. Peaks in the mass spectra of extracts were fragmented by ETD and subsequently identified.[22] Sarsby et al. applied automated LESA MS/MS for the analysis of intact protein biomarkers of nonalcoholic liver disease.[23]

Here, we present LESA mass spectrometry of intact proteins directly from *Escherichia coli* colonies growing on solid agar in Petri dishes. The LESA sampling system was coupled to a high mass resolution orbitrap mass spectrometer. Optimization of the surface sampling process revealed that a modified surface sampling routine, in which the pipet tip came into contact with the colony surface, resulted in detection of greater numbers of protein signals. This sampling process may offer the additional advantage of limiting the spread of solvent to an area the size of the pipet tip, which has implications for the spatial resolution of any images produced. In order to identify proteins, ions corresponding to intact proteins were selected and subjected to CID. The resulting MS/MS data were searched against an *E. coli K-12* protein database using Prosight PTM 2.0 software. Six proteins were identified including DNA binding proteins and shock response proteins.

■ EXPERIMENTAL SECTION

Materials. A strain of *Escherichia coli K-12* was inoculated onto solid LBA medium (LB 20 g L^{-1}, agar 20 g L^{-1}) in 6 cm diameter Petri dishes. The smaller size of Petri dish allows it to fit inside the sample tray of the Advion TriVersa NanoMate system. Bacteria were incubated at 21 °C for 3 days before storing in the dark at 4 °C or were allowed to grow at room temperature on the bench and were stored at room temperature (~20 °C). The solvent system for surface sampling/electrospray ionization consisted of acetonitrile (J.T. Baker, The Netherlands) and water (J.T. Baker, The Nether-

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lands) (39.6:59.4) with 1% formic acid (Sigma-Aldrich Company Ltd., Dorset, U.K.).

Surface Sampling. Petri dishes containing *E. coli K-12* colonies were placed directly into the TriVersa Nanomate chip-based electrospray device (Advion, Ithaca, NY) adjacent to half a 96-well microtiter plate (ABgene PCR plate Thermo Fisher Scientific, Leicestershire, U.K.) into which the extraction solvent system is placed; see Figure 1. The electrospray device was coupled to a Thermo Fisher Orbitrap Velos ETD (Thermo Fisher Scientific, Bremen, Germany) instrument. Surface sampling was performed using the advanced user interface (AUI) feature of the TriVersa NanoMate ChipSoft Manager software which controls the TriVersa NanoMate robotic system. A coordinate (x,y,z) system was used to direct the robotic arm in a surface sampling routine. The arm picked up a conductive pipet tip from the tip rack and relocated to a position above the well containing extraction solvent. The tip descended into the well and aspirated 3 μL of solvent. The arm relocated to a defined position above the bacterial colony; the tip descended to a depth just below the surface of the colony. The exact sampling height varied (from −9 to −11 mm from the initial position of the robotic arm), depending on the size of the colony and spread of agar in different samples; for increased extraction of proteins, the pipet tip came into contact with the top surface of the colony but did not penetrate the agar below. Once in contact with the colony, the tip dispensed 2 μL of solvent onto the colony which was held in contact for 3 s to allow soluble analytes to desorb into the droplet. The solution was then reaspirated into the pipet, and the tip engaged with the nanoelectrospray chip in order to start electrospray ionization and introduction of the sample into the mass spectrometer.

Mass Spectrometry. All mass spectrometry experiments were performed on a Thermo Fisher Orbitrap Velos ETD (Thermo Fisher Scientific, Bremen, Germany) instrument. The sample was introduced via the Triversa Nanomate with a gas pressure of 0.3 psi and a tip voltage of 1.75 kV. Mass spectra were collected in full scan mode (m/z 500−2000) at a resolution of 100 000 at m/z 400. The AGC target was 1×10^6 charges. Each scan was composed of 30 coadded microscans. Data were recorded for 5 min (∼11 scans). (Note that a stable electrospray could be maintained for ∼50 min from a single LESA analysis.) CID was carried out in the linear ion trap, and the fragment ions were detected in the orbitrap at a resolution of 100 000 at m/z 400. AGC target was 5×10^4 charges. CID experiments were performed with helium gas at normalized collision energy of 35% (optimized for sequence coverage, data not shown) and an isolation width of 10 Th. MS/MS spectra were collected in the range of m/z 300−2000 and are composed of 10 coadded microscans. Data were analyzed using Xcalibur 2.10 software (Thermo Fisher Scientific) where the Xtract program was used to calculate monoisotopic masses. For data analysis using ProSightPTM 2.0, CID MS/MS spectra were deconvoluted using the Xtract program (S/N threshold 3). Xtracted monoisotopic fragment masses were searched against the *E. coli K-12* database in ProSightPTM 2.0, with a fragment tolerance of 10 ppm and precursor tolerance of 100 ppm, in order to assign putative protein identifications. Two search types were used in Prosight, "absolute mass search" and "sequence tag search". Absolute mass search involves searching the mass of the precursor ion against intact masses of proteins within the chosen database. If a match is found, then fragment masses can be matched to theoretical fragments of that protein. A sequence tag search involves compiling lists of amino acids which could correspond to observed fragment masses or differences in mass between two fragments. The amino acid sequences are then searched against the appropriate database to see if they correspond to sequences of known proteins. Protein identities were confirmed by manual analysis using Protein Prospector (http://prospector.ucsf.edu/prospector/mshome.htm).

Postsampling Image Analysis. Photographs of the colony surface were taken after LESA sampling. Areas which came into contact with solvent during sampling were measured using ImageJ software. Contact with solvent caused discoloration of the colony surface so the diameter of these regions could be visually identified in the photos. The image scale was set using features of known size in the image, either a ruler or coordinate spots of the universal adaptor plate.

RESULTS AND DISCUSSION

Optimization of LESA Method. When setting up a LESA method, there are several parameters which are user-defined and can affect the extraction efficiency including the solvent system used for extraction and the height to which the pipet descends above the sample surface. These parameters were optimized for extraction of intact proteins from bacteria. A survey of common LESA solvent systems was conducted (data not shown) and indicated that an acetonitrile/water mixture was the only solvent capable of extracting proteins. The optimum pipet tip height above the surface was investigated by sequentially sampling the surface, starting at approximately 0.2 mm above the surface, allowing the tip to descend 0.2 mm lower each time and recording spectra at each respective sampling height; see Supplementary Figure 1, Supporting Information. (Mass spectra shown in Supplementary Figure 1, Supporting Information, were recorded from the same location.) It was found that proteins were extracted when the pipet tip came into contact with the colony surface. (Peaks observed in the mass spectrum shown in Supplementary Figure 1a, Supporting Information, correspond to singly charged species.) Although a larger number of proteins were detected when the pipet tip sampled further below the colony surface (see Supplementary Figure 1c, Supporting Information), a compromise must be made between good extraction of proteins and electrospray instability due to capillary blocking. The mass spectrum shown in Supplementary Figure 1d, Supporting Information, contains peaks corresponding to singly, doubly, and triply charged species indicating that the pipet tip may have punctured the agar below the colony. For comparison, a mass spectrum obtained following LESA sampling of agar is shown in Supplementary Figure 2, Supporting Information. We hypothesize that the observation of increased numbers of proteins in mass spectra obtained following contact between the pipet tip and the surface is due to the insoluble nature of the extracellular matrix produced by colony bacteria; in placing the pipet tip in contact with the surface, this insoluble layer is physically disrupted allowing more efficient extraction of proteins contained within the colony. It should be noted that this "contact" method of surface sampling is different from other LESA methods described in the literature; see Figure 2a,b. Kai et al. report the extraction of small molecule antibiotic compounds using LESA, a method in which only the solvent droplet came into contact with the bacteria.[18]

An interesting consequence of the "contact" surface sampling routine is illustrated in Figure 2. The process of placing the

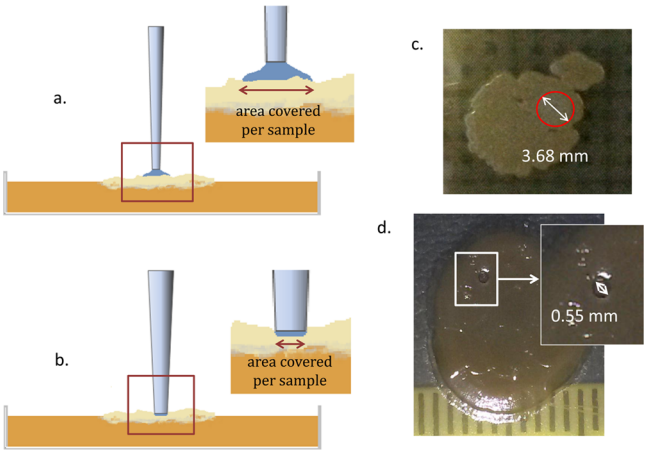

Figure 2. Improved sampling precision obtained from "contact" LESA sampling: (a) Schematic illustrating the surface area of sample covered by each solvent droplet when sampled by a liquid microjunction (contact between surface and solvent only); (b) schematic illustrating the smaller surface area covered by each sample droplet with the "contact" method (contact between surface and pipet tip); (c) sampled area following liquid microjunction sampling; (d) sampled area following "contact" LESA.

pipet tip in contact with the colony surface during sampling appears to limit the spread of the solvent droplet to the area of the pipet tip end. We postulate that the low pressures used to dispense and aspirate the solvent droplet are insufficient to push the solvent beyond the end of the pipet tip and therefore the colony acts as a plug. The spread of solvent during LESA sampling determines the area of the sampling region. In the case of hydrophilic surfaces, the area over which the microjunction spreads is large; see Figure 2a,c. The method described here prevents any solvent spread beyond the edges of the pipet tip because the solvent is completely encased within the end of the tip; see Figure 2b,d. The total surface area sampled by this method is ~0.24 mm^2, as measured using ImageJ on images of the puncture mark on the colony surface after sampling. This observation represents a significant improvement when compared to the area covered by the usual LESA solvent droplet, which was measured to be ~10.64 mm^2 (solvent sampling discolored the colony surface allowing for measurement of the area from photographic images). Note that the droplet volumes were the same for both methods.

Top-Down LESA Mass Spectrometry of *E. coli* Colonies. Figure 3a shows a representative full scan mass spectrum obtained following LESA sampling of an *E. coli* colony (previously stored at 4 °C) growing on agar; see Figure 3b for the location of the spot sampled. The mass spectrum contains approximately 150 peaks corresponding to ~60 bacterial proteins in a range of charge states. The molecular weights of these proteins ranged from approximately 5 to 32 kDa. This mass range is in agreement with the findings of McFarland et al., who report the detection of intact proteins in the range of 4–37 kDa from *S. typhimurium* by ESI orbitrap mass spectrometry.[12]

Figure 3c shows an enlarged section of the mass spectrum with peaks selected for CID indicated. Supplementary Figure 3, Supporting Information, shows the same region of the mass spectra obtained following LESA sampling at various locations as indicated (Figure 3c is also Supplementary Figure 3c, Supporting Information). The relative abundance of the protein peaks is greater in the mass spectra obtained from locations close to the edge of the colony. That observation may be the

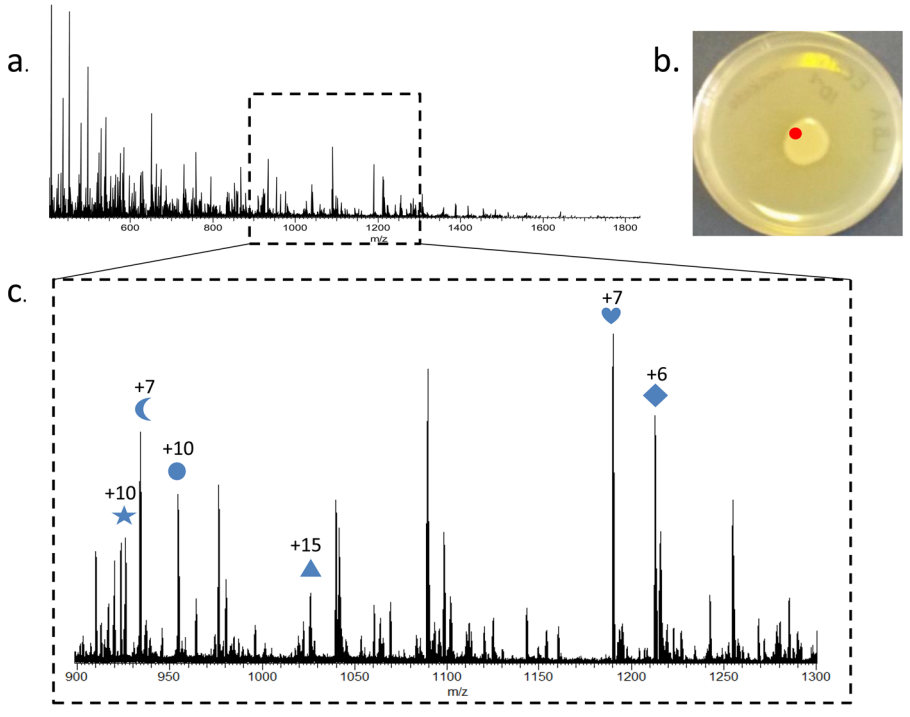

Figure 3. LESA mass spectrometry of an *E. coli K-12* bacterial colony previously stored at 4 °C. (a) Full scan mass spectrum; (b) photograph of bacterial colony growing on solid agar medium in a Petri dish. Red spot marks the region sampled by LESA; (c) enlarged m/z region from a full scan mass spectrum (m/z 900–1300) which contains the majority of peaks corresponding to protein ions. Symbols indicate peaks subsequently selected for CID.

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Table 1. Summary of Proteins Identified from *E. coli K-12* Colonies Following "Contact" LESA Top-Down Mass Spectrometry

m/z_{meas}	m/z_{calc}	Δppm	charge state	MW_{calc}	% sequence coverage	protein identity	protein function
954.5104	954.5133	−3.0	+10	9530.24	28	DNA binding protein HU-alpha	stabilizes DNA under extreme environmental conditions
1027.6038	1027.6069	−3.0	+15	15399.95	18	DNA binding protein H-NS	increases thermal stability of DNA
1189.5921	1189.5919	0.2	+7	8321.10	23	UPF0337 protein YjbJ	predicted shock response protein
1212.2801	1212.2719	6.8	+6	7268.60	13	CspA	cold shock response protein
923.0039	923.0067	−3.0	+10	9221.00	40	DNA-binding protein HU-beta	stabilizes DNA under extreme environmental conditions
933.4871	933.4822	5.2	+7	6528.35	32	multiple stress resistance protein BhsA	inhibits biofilm formation by repressing cell aggregation

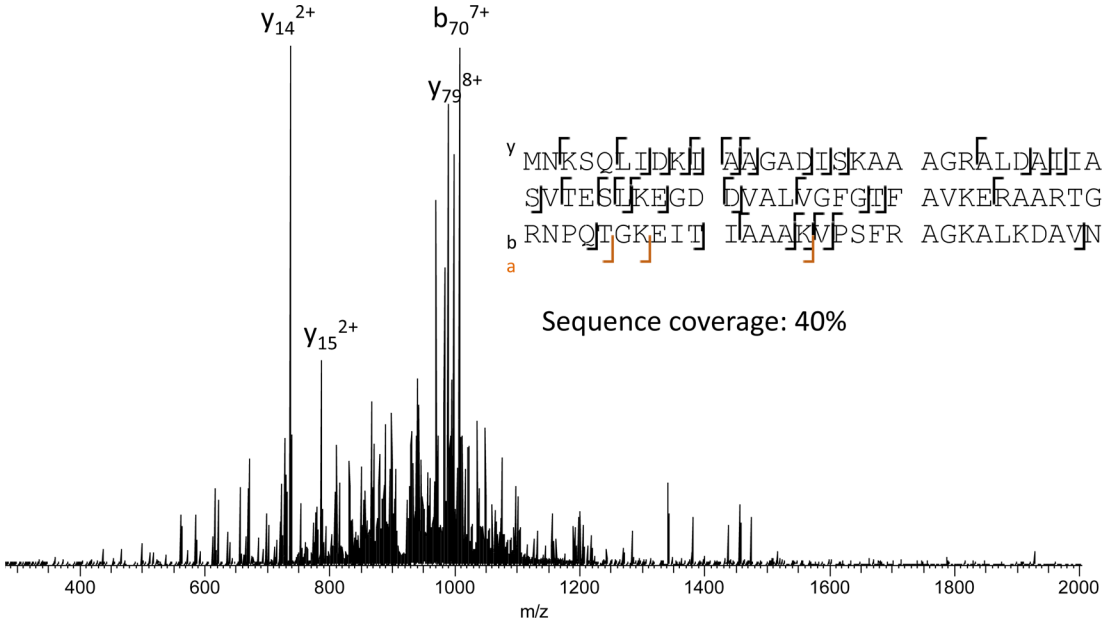

Figure 4. CID mass spectrum of ions centered at m/z 923.51 (+10 charge state), identified as DNA-binding protein HU-beta. Fragment ions observed are shown the inset.

result of easier disruption of the extracellular matrix and improved extraction efficiency at the colony edge.

Ions centered at m/z 923.51 (+10 charge state), 933.91 (+7), 954.43 (+10), 1028.14 (+15), 1190.31 (+7), and 1212.94 (+6) were fragmented. The product ions were searched against the *E. coli K-12* database in ProSightPTM 2.0, using either the "absolute mass search" or the "sequence tag search". Putative protein identifications were confirmed by manual analysis. The proteins identified and the protein sequence coverages are shown in Table 1. The sequence coverages ranged from 13% to 40%. A representative MS/MS spectrum from one of the identified proteins, DNA-binding protein HU-beta, is shown in Figure 4. The remaining MS/MS spectra are shown in Supplementary Figures 4−8, Supporting Information. Product ion assignments are shown in Supplementary Tables 1−6, Supporting Information.

A total of 6 proteins were identified. DNA binding protein HU-alpha and HU-beta are both histone-like DNA binding proteins which are capable of wrapping DNA to stabilize and prevent its denaturation under extreme environmental conditions.[24] DNA binding protein H-NS increases thermal stability of DNA and inhibits transcription at low temperatures.[25] The function of protein YjbJ is poorly understood but is recognized as a highly abundant protein in *E. coli*.[26] CspA is a cold shock response protein, known to be induced at temperatures of 15 °C or lower.[27] Multiple stress resistant protein BhsA is known to be induced under a range of stressful conditions including drastic pH changes, heat shock, the presence of heavy metals, and hydrogen peroxide. As a result, this protein inhibits biofilm formation by repressing cell aggregation and increases cell viability under stressful conditions.[28] A number of these proteins (HU-alpha, HU-beta, YjbJ, H-NS) were also detected by ESI top-down MS/MS of *S. typhimurium* reported by McFarland et al.

It should be noted that, while a number of proteins have been identified in this study, it was not possible to identify all proteins from their MS/MS spectra. Ions of m/z 1254.99 (+7 charge state) were also fragmented, and the product ions were searched against the *E. coli K-12* database in ProSightPTM 2.0, using both the "absolute mass search" and the "sequence tag search". Neither search returned hits corresponding to the correct intact mass. Top-down methods are used infrequently relative to bottom-up identification techniques, and as such, the demand for software for identification of proteins by top-down methods is lower. While several platforms are available, e.g., Prosight PTM 2.0,[29] ROCCIT (roccit.caltech.edu), the proteins in this work were not reliably fully characterized and a large amount of manual characterization was required. This aspect would need to be addressed if top-down methods were to become more established.

The selection of proteins detected and identified from colonies stored at 4 °C is indicative of extreme environmental

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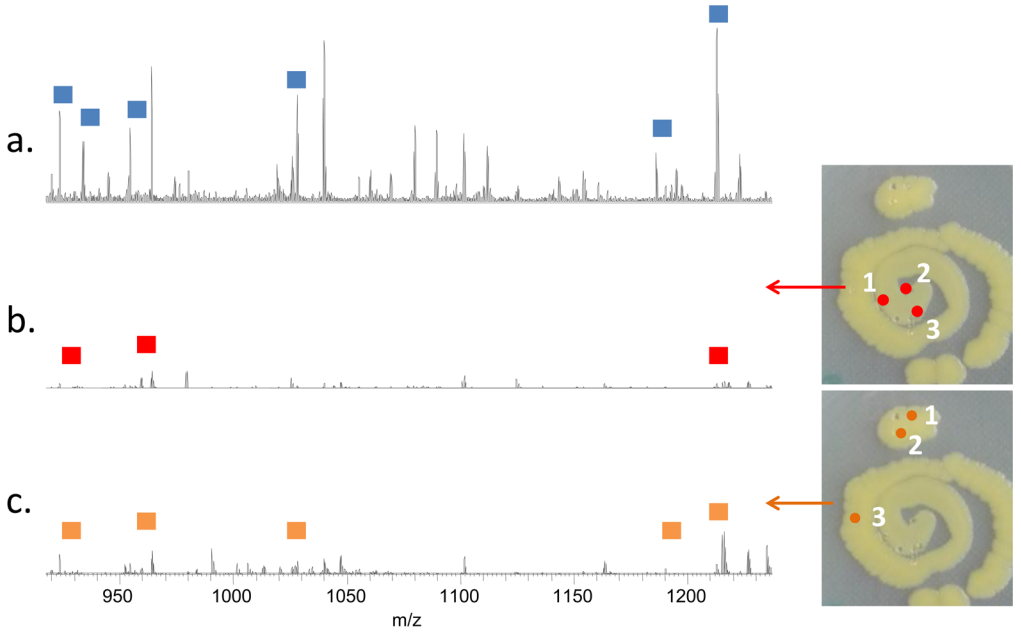

Figure 5. LESA mass spectra of bacteria stored at (a) 4 °C for 2 days, (b) at room temperature (20 °C) for 3 days, and (c) at room temperature (20 °C) for 10 days. (*y*-axes are equivalent.) Symbols denote identified stress response proteins. Inset: Photographs of colonies with sampling locations indicated.

conditions, consistent with prolonged refrigerated storage conditions. After the initial incubation period to grow the colonies, samples were stored at 4 °C for a number of days, which explains the induction of a cold shock response. For comparison, a further set of bacterial samples were grown. These colonies were stored at room temperature (~20 °C) prior to mass spectrometric analysis and were never exposed to low (refrigerated) temperatures.

Figure 5 shows LESA mass spectra obtained from bacteria grown and stored at room temperature. For comparison, a LESA mass spectrum from a colony grown at room temperature for 3 days and then stored at 4 °C for 2 days is shown in Figure 5a. Peaks corresponding to all the proteins identified from the LESA mass spectrum shown in Figure 3 (in which bacteria had been grown for 3 days at room temperature before being stored at 4 °C for 7 days) were observed, albeit with differing relative abundances. That may be due to differences in protein expression as a result of prolonged cold shock or may be due to differences in LESA extraction efficiency either as a result of prolonged cold shock or colony topology. Figure 5b shows a mass spectrum obtained from a colony stored at room temperature for 3 days. The colony was sampled at three separate locations, as indicated on the photograph inset. (Individual mass spectra from each location are shown in Supplementary Figure 9, Supporting Information.) At location 1, peaks corresponding to HU-alpha and HU-beta were observed, but none of the other proteins identified above were seen. At location 2 (Figure 5b), peaks corresponding to HU-alpha, HU-beta, and CspA were observed. At location 3, none of the proteins identified above were observed. The proteins BhsA and YjbJ were not observed in any location.

A repeat analysis was carried out after the colonies had been stored at 20 °C for a further 7 days (Figure 5c). Again, three separate locations were analyzed, as indicated. (Individual mass spectra obtained from each location are shown in Supplementary Figure 10, Supporting Information.) In two of the locations (2 and 3), all but one (BhsA) of the stress response proteins previously detected in bacteria stored at 4 °C were detected. At location 1, all but BhsA and YjbJ were detected. These results suggest that prolonged storage can induce various stress responses in bacteria. Multiple stress resistance protein BhsA, however, was not detected at any location from colonies stored at 20 °C, either after 3 or 10 days, suggesting that either temperatures lower than 20 °C or storage for longer than 10 days is required for the induction of this protein. As mentioned above, BhsA has been shown to inhibit biofilm formation. It is possible that expression of BhsA under refrigerated conditions resulted in reduced biofilm formation thus aiding the LESA process.

CONCLUSIONS

We have demonstrated a method suitable for the top-down extraction, detection, and identification of intact proteins from living bacterial colonies. The identities of these proteins appear to indicate the well-being of the colony. The method has implications for microbiological research as it may be suitable for the study of bacterial growth, communication, and response to external factors such as pharmaceuticals and pH. Moreover, the "contact" LESA method offers improved sampling precision and could be useful for spatial profiling of bacterial colonies or analysis of contaminated surfaces.

ASSOCIATED CONTENT

Supporting Information

LESA mass spectra, LESA CID MS/MS spectra, and CID fragment assignments. This material is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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