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Top-down proteomic identification of bacterial protein biomarkers and toxins using MALDI-TOF-TOF-MS/MS and post-source decay

Abstract: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is a new tool for rapid identification of bacterial microorganisms. With the development of matrix-assisted laser desorption/ionization time-of-flight-time-of-flight (MALDI-TOF-TOF) instruments, new capabilities for characterizing bacteria and their protein biomarkers and toxins by top-down proteomic identification is now possible. A number of researchers have demonstrated top-down proteomic identification of nondigested proteins using MALDI-TOF-TOF platforms. The current review briefly discusses the use of MS instrumentation for bacterial identification and characterization as well as recent developments in the use of MALDI-TOF-TOF for top-down proteomic identification of protein biomarkers and toxins from unfractionated bacterial cell lysates. This approach provides great promise as a rapid method for identification and characterization of pathogenic microorganisms and their virulence factors.

Keywords: matrix-assisted laser desorption/ionization time-of-flight-time-of-flight (MALDI-TOF-TOF); post-source decay; top-down proteomic identification.

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Introduction: bacterial identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has become an increasingly popular analytical tool for rapid identification and characterization of microorganisms (Fenselau and Demirev 2001, Lay 2001, Demirev and Fenselau 2008, Rajakaruna et al. 2009). Its popularity stems from a number of factors: simplicity of sample preparation, speed of data acquisition and analysis, relative ease of instrument training, and availability of commercial software packages for data analysis. All of these factors (and others) combine to allow MS experts and nonexperts to exploit this technology for applied and fundamental research in microorganisms: bacteria, viruses, and fungi.

The success of the MALDI-TOF-MS approach for bacterial identification is attributable to detection of a small number (20–100) of highly abundant, low-molecular-weight (5–20 kDa) proteins ionized from unfractionated bacterial cell lysates or intact bacterial cells. As ionization by MALDI results in primarily singly charged protein ions, peak congestion is not a significant concern and charge state deconvolution is not required (Karas et al. 1987, Tanaka et al. 1987). Because of the low charge states generated by MALDI, a mass analyzer having an unlimited mass range is most compatible, e.g., TOF-MS. As an ionization technique, MALDI is biased toward ionization of high copy, lower MW proteins. This intrinsic bias is enhanced when ionizing from a protein mixture. In consequence, only high copy, relatively low MW proteins are ionized from an unfractionated bacterial cell lysate. There are many more proteins present in an unfractionated, undigested bacterial cell lysate than are actually ionized by MALDI.

Another groundbreaking ionization technique, electrospray ionization (ESI) (Fenn et al. 1989), is not feasible for the analysis of an unfractionated mixture because ESI generates protein ions at higher charge states, resulting in peak congestion and complicating data deconvolution (not to mention ionization suppression). In consequence, protein mixtures, as found in bacterial cell lysates, must be fractionated, usually by liquid chromatography (LC), before ionization by ESI. LC coupled with ESI-MS on a hybrid quadrupole-TOF mass spectrometer has been successfully developed to analyze bacterial cell lysates (Williams et al. 2002). The multiple charge state envelopes of protein ions from LC-ESI/MS are deconvoluted in 30- to 60-s segments of retention time and then combined to create a single composite spectrum of protein molecular

weight (MW) vs. intensity. This novel approach is quite attractive because the number of proteins detected is significantly greater (up to 500) than that observed in an unfractionated sample analyzed by MALDI (Williams et al. 2002). The greater number of proteins ionized and detected allows greater bacterial taxonomic resolution, thus allowing more closely related microbial strains to be distinguished. One drawback is the time required for LC separation (60–90 min) and data deconvolution. In addition, it may be necessary to perform 3–5 replicate LC analyses to confirm the presence of strain-specific protein biomarkers. By contrast, replicate analyses by MALDI-TOF-MS takes only seconds.

A number of commercial (Stephan et al. 2011, Ngan et al. 2012, van Belkum et al. 2012) and in-house software programs (Jarman et al. 2000) have been developed that use pattern recognition algorithms for bacterial identification by MALDI-TOF-MS. The pattern recognition approach identifies bacteria from the "pattern" or "fingerprint" generated from the mass-to-charge (m/z) and intensity of a series of protein ions observed in an MS spectrum. The identity of specific protein ions is not relevant to bacterial identification. A bacterial identification is made from a comparison of the MS spectrum of an unknown bacteria strain to MS spectra of known reference bacteria strains. A variety of algorithms has been developed to score/rank unknown-to-reference comparisons. The most significant "match" (e.g., low p-value) above a set threshold is considered an identification.

A number of factors can affect the intensity of protein ions by MALDI: sample preparation, MALDI matrix, instrument tuning, and performance. In consequence, pattern recognition algorithms typically use only the m/z of protein ions for analysis (although software applications may allow the user to include peak intensity as part of the analysis criteria). Pattern recognition identification is very dependent on MS reproducibility. A potential weakness of this approach is that the quality of MS data can vary considerably between instruments and laboratories. In consequence, sample preparation and instrument-tuning protocols must be rigorous to obtain reproducible results.

The MALDI-TOF-MS approach has been most successful with researchers who create an MS reference database using their own in-house strain collections. Although there are commercially available reference databases, using such libraries require, for optimum performance, the same sample preparation protocol used to generate the reference library (Ngan et al. 2012). A stand-alone pattern recognition software that is not linked to any particular MALDI-TOF platform is commercially available (Stephan et al. 2011).

Using the pattern recognition approach, bacteria have been identified at the genus, species, subspecies, and, in some cases, strain level (Mandrell et al. 2005, Fagerquist et al. 2006, Sandrin et al. 2012). As MALDI-TOF detects differences in protein mass, its ability to provide the desired taxonomic "resolution" is dependent upon amino-acid variations among proteins that are ionized by MALDI from an unfractionated sample. These amino-acid variations, in turn, are dependent upon gene variations across genera, species, subspecies, and strains. The greater the genetic diversity of a bacteria, the more likely that MALDI-TOF will provide the desired taxonomic "resolution". However, if a bacteria has a high degree of genetic similarity, then the resulting protein sequence homology will make it challenging to use MALDI-TOF to discriminate at the finer taxonomic detail. The critical question is whether MALDI-TOF-MS provides the level of taxonomic resolution necessary to address a specific application of the researcher.

With the emergence of genomic sequencing, it became possible to link the theoretical MWs of open reading frames (ORF) of sequenced bacterial genomes to the m/z of protein biomarkers of MALDI-TOF-MS (Demirev et al. 1999, Pineda et al. 2000). If a sufficient number of protein ions are "matched" to *in silico* protein MWs from a genome, the bacteria are considered identified. The significance of the identification is calculated using a p-value algorithm (Demirev et al. 1999, Pineda et al. 2000). The most common post-translational modification (PTM) of bacterial proteins is the removal of the N-terminal methionine (N-Met) that in most cases follows a predictive biological rule based upon the identity of the penultimate residue (Hirel et al. 1989, Gonzalez and Baudouy 1996, Solbiati et al. 1999). This rule was further incorporated into analysis when calculating the *in silico* protein MWs of a genome (Demirev et al. 1999, Pineda et al. 2000). This bioinformatics approach to microorganism identification has a number of advantages. First, an identification is less dependent on stringent sample preparation, instrument tuning, and performance because an MS spectrum is compared with *in silico* MWs in a sequenced genome not to another MS spectrum. Second, unlike pattern recognition analysis, where the identity of specific protein ions are not relevant to microorganism identification, a bioinformatic-based bacterial identification assumes that protein ions with a corresponding ORF "match" are also tentatively assigned. However, "assignment" does not mean the protein ion is identified. Protein ion identification requires isolation and sequence-specific fragmentation. An important caveat of this approach is that a bacterial strain (or closely related strain) must be genomically

sequenced to be identified. However, with the development of next-generation DNA sequencers, it is foreseeable that any unknown bacterial strain could be identified either from a closely related sequenced strain already in public databases or from the rapid sequencing and annotation of the strain itself.

MALDI-TOF-TOF-MS/MS and top-down identification of bacterial proteins

The development of time-of-flight-time-of-flight (TOF-TOF) mass analyzers has significantly advanced peptide and protein analysis by MALDI (Medzihradszky et al. 2000, Suckau et al. 2003, Cotter et al. 2007). MALDI-TOF-TOF was initially developed for high-throughput tandem mass spectrometry (MS/MS) of peptides of digested proteins (i.e., "bottom-up" proteomics; Washburn et al. 2001, Aebersold and Mann 2003), but it was quickly demonstrated that small or modest-sized, nondigested protein ions could also be analyzed by MS/MS to allow a top-down proteomic identification (Lin et al. 2003, Demirev et al. 2005, Liu and Schey 2008, Fagerquist et al. 2009, 2010). As the ions generated by MALDI are primarily (although not exclusively) singly charged, the fragment ions are also singly charged, which greatly simplifies data interpretation.

Top-down proteomic analysis has been primarily the domain of high-resolution and high-mass accuracy mass analyzers, e.g., Fourier transform ion cyclotron resonance (Wang and Marshall 1989) or Orbitrap™ (ThermoFisher, San Jose, CA, USA) (Hu et al. 2005). Typically, these instruments use ESI and novel dissociation techniques, e.g., electron-capture dissociation (Zubarev et al. 1998) and electron transfer dissociation (ETD) (Syka et al. 2004). As ESI generates multiply charged protein ions, high resolution and mass accuracy are critical for data deconvolution and analysis. Software has also been developed for top-down proteomic analysis to facilitate the interpretation of information-rich MS/MS spectra obtained on such instruments (Leduc and Kelleher 2007, Durbin et al. 2010).

Three different approaches have been developed for top-down proteomic identification using MALDI-TOF-TOF instruments. The first approach exploits protein ion fragmentation that can occur in the source during and after the desorption/ionization laser pulse but before ions are accelerated from the source and is referred to as in-source decay (ISD) (Suckau and Resemann 2003, Hardouin 2007, Calligaris et al. 2010). This is considered to be a fast fragmentation process likely the result of electron capture by a multiply protonated analyte cation (i.e., recombination) that can occur in the expanding plume during desorption/ionization laser pulse. This type of fragmentation results in polypeptide backbone cleavage near the C- and/or N-termini resulting in fragment ions with a mass of 1 to 4 kDa. The fragment ions generated may be sufficient for peptide mass fingerprinting, resulting in a searchable sequence "tag" that, when combined with the mass of the protein, can be used to identify the protein from a database. ISD fragment ions can be further mass-selected and fragmented and the fragment ions analyzed, i.e., MS/MS, using either collision-induced dissociation (CID) (Liu and Schey 2008) or laser-induced dissociation (LID) (Spengler 1997, Lin et al. 2003, Demirev et al. 2005, Fagerquist et al. 2009, 2010). MS/MS of ISD fragment ions provides further sequence coverage extending to the N- or C-termini. This approach has been referred to as "T^3-sequencing" by its practitioners (Calligaris et al. 2010). As the original protein ion is fragmented in the source without prior ion selection/isolation, the ISD approach requires a pure protein and is not feasible for the identification of a protein in a mixture of proteins, e.g., unfractionated bacterial cell lysate, as one cannot know that a specific fragment ion came from which protein precursor ion.

The second approach involves true MS/MS where the precursor ion is mass selected/isolated by the first TOF and then undergoes fragmentation by CID, and the resulting fragment ions are analyzed by the second TOF. This approach works most effectively with multiply charged protein ions, e.g., doubly and triply charged protein ions. Liu and Schey (2008) demonstrated top-down identification of pure protein standards by CID of doubly and triply charged ions using α-cyano-4-hydroxycinnamic acid (CHCA) matrix. Because of its higher ionization energy, CHCA is more likely to generate multiply charged protein ions compared with other MALDI matrices (Frankevich et al. 2003).

The third approach (and that used by the author) involves MS/MS and LID-PSD (Spengler 1997, Lin et al. 2003, Demirev et al. 2005, Liu and Schey 2008, Fagerquist et al. 2009, 2010). LID is somewhat confusing terminology because it actually refers to an ion that fragments after exiting the source, i.e., postsource decay (PSD), as a result of energy deposited into the molecule/ion during the desorption/ionization laser pulse or due to collisions between analyte and matrix molecules in the source. Traditionally, this has been referred to as unimolecular dissociation or metastable decay (Cotter et al. 2007).

LID-PSD is a slow fragmentation process and should not be confused with photofragmentation, i.e., direct bond cleavage of a mass-selected ion by a synchronized laser pulse orthogonal to the ion beam at the collision cell (Zhang and Reilly 2010). As ISD and LID-PSD occur in different regions of the mass spectrometer, the internal energies of metastable protein ions, their dissociation channels, and the fragment ions generated are very different. LID-PSD occurs *via* lower-energy dissociation channels compared with ISD. In consequence, PSD does not result in polypeptide backbone cleavages at a series of adjacent residues at the C- or N-termini. Instead, the fragment ions generated (a, b, and y types and small neutral losses of water and ammonia) are primarily, but not exclusively, the result of polypeptide backbone cleavage at the C-terminal side of aspartic acid (D) and glutamic acid (E) residues and at the N-terminal side of proline (P) residues (Lin et al. 2003). The transfer of the acidic proton from the side-chain of E and D residues to the polypeptide backbone facilitates cleavage at these residues. Cleavage at proline residues is due to the 90° turn of the polypeptide backbone at this residue. Because the D, E, and P residues are distributed randomly in a protein sequence, the range of fragment ion m/z can be wide: m/z 1000 up to the precursor ion m/z. However, it is beyond the capability of the second TOF analyzer to isotopically resolve the higher m/z fragment ions, and in addition, the most abundant isotopomer is not the monoisotopic isotopomer. In consequence, MS/MS-PSD data must be processed before it is compared with the average m/z of *in silico* fragment ions for top-down identification (Fagerquist et al. 2009, 2010).

The sequence-specific fragment ions generated by MS/MS-PSD can be used to identify a protein (and its source organism) by comparison to a database of *in silico* fragment ions of proteins having the same MW as the protein being analyzed (Lin et al. 2003, Demirev et al. 2005, Liu and Schey 2008, Fagerquist et al. 2009, 2010). One important advantage of the MS/MS-PSD approach is that proteins in a mixture can be analyzed (e.g., unfractionated bacterial cell lysate) because a protein ion is mass selected/isolated before it is fragmented, i.e., true MS/MS, and thus, specific fragment ions can be attributed to specific precursor ions.

Demirev et al. (2005) were the first to demonstrate top-down proteomic identification of a microorganism using the MALDI-TOF-TOF-MS/MS using LID-PSD. Small acid-soluble proteins (SASPs) from *Bacillus atrophaeus* and *Bacillus cereus* were analyzed from pure and mixed culture samples by MS/MS-PSD. Sequence-specific fragment ions allowed top-down identification of the SASP and the microorganism by comparison to an *in silico* database of all bacterial proteins having the same MW as the protein ion. A p-value algorithm was developed to score/rank identifications using software developed in-house (Demirev et al. 2005).

Our laboratory has been using MALDI-TOF-TOF-MS/MS-PSD and Web-based software (developed in-house at the USDA) for rapid top-down identification of protein biomarkers and toxins from food-borne pathogens (Fagerquist et al. 2009). Nondigested proteins are ionized from unfractionated bacterial cell lysates. Our Web-based software uses a simple peak-matching algorithm as well as incorporating Demirev's p-value to independently score/rank identifications. As metastable protein ions typically undergo PSD at specific residues, our software allows the option of comparing MS/MS fragment ions to *in silico* fragment ions that are adjacent to specific residues, e.g., D, E, P residues. This residue-specific analysis can increase the score differential between the top-ranked (correct identification) and a second-ranked (incorrect identification) compared with the score differential obtained using a non-residue-specific analysis. Our software also allows an identification to be confirmed by fragment ion error analysis (i.e., the difference between the observed and the theoretical fragment ion m/z). Systematic fragment ion error associated with a correct identification and due to instrument calibration drift can be fitted to a line, whereas an incorrect identification should display random fragment ion error (Fagerquist et al. 2009).

We have used this approach to identify protein biomarkers of *Campylobacter* (Fagerquist et al. 2009) and pathogenic and nonpathogenic strains of *Escherichia coli* (Fagerquist et al. 2010). The power of the MS/MS-PSD approach is particularly apparent for the YahO protein of *E. coli* where a single amino-acid substitution (D↔N) results in a significant change in protein ion fragmentation that can be used to distinguish a pathogenic strain from a nonpathogenic strain (Fagerquist et al. 2010).

To increase MALDI-TOF-TOF mass accuracy for MS/MS-PSD analysis of nondigested proteins, a calibrant was also developed that uses the average m/z of five prominent PSD fragment ions of reduced/alkylated thioredoxin (AlkTrx) (Fagerquist and Sultan 2012). These calibrant fragment ions span a higher m/z range than that of fragment ions from peptide calibrants. In a side-by-side comparison, the AlkTrx calibrant demonstrated increased mass accuracy and more significant top-down identifications compared with using the monoisotopic fragment ions of a peptide calibrant ([Glu^1]-fibrinopeptide B).

We have also extended this technique to the top-down identification of the Shiga toxin 2 or Stx2 (an AB_5 toxin) and its sequence-specific variants from Shiga toxin-producing *E. coli* (Fagerquist and Sultan 2010, Fagerquist and Sultan 2011). *E. coli* O157:H7 (strain EDL933) was exposed to a DNA-damaging antibiotic (ciprofloxacin) eliciting the bacterial SOS response leading to over-expression of bacteriophage-encoded elements including Stx2 (Johannes and Romer 2010). We identified the B-subunit and the A2 fragment of the A-subunit from an unfractionated bacterial cell lysate. The mature B-subunit (~7.8 kDa) has two cysteine residues that are involved in an intramolecular disulfide bond creating a large "looped" secondary structure. MS/MS-PSD of the B-subunit reveals several fragment ion triplets (with the center fragment ion flanked by two fragment ions at m/z ±33), which are the result of cleavage of the polypeptide backbone between the two cysteine residues and the symmetric and asymmetric cleavage of the disulfide bond. As the fragment ions corresponding to asymmetric cleavage of the disulfide bond are not present in our *in silico* database, these fragment ions could not be correctly assigned and their presence reduced the significance of the correct identification. However, MS/MS-PSD of the disulfide-reduced sample eliminated fragment ions from asymmetrically cleaved disulfide bond while simultaneously increasing the fragmentation efficiency of the now linearized polypeptide backbone resulting in an improved top-down identification. It should be noted that the fragment ions from asymmetric cleavage of the disulfide, although complicating sequence-specific analysis, do provide critical information of an important PTM in the mature Stx2 B-subunit. The mass of the catalytically active A1 fragment (~28 kDa) of the A-subunit precluded its detection in an unfractionated bacterial cell lysate; however, the smaller A2 fragment (~5.2 kDa) was ionizable after A2 was enzymatically cleaved from A1 using furin (a membrane-bound endopeptidase). As the greatest sequence variability in variants of Stx2 occur in the B-subunit and the A2 fragment, top-down of A1, although desirable, is usually not critical to detecting and distinguishing sequence-specific variants of Stx2.

Antibiotic susceptibility testing (AST) is a well-established microbiological technique to measure bacterial resistance to specific antibiotics from changes in bacterial growth during culturing in the presence of such antibiotics (Cook et al. 2011). At a molecular level, bacterial antibiotic exposure can trigger specific genes (e.g., SOS response) resulting in changes in protein expression that can be analyzed by MALDI-TOF-TOF-MS/MS-PSD. Top-down proteomic identification by MS/MS-PSD is likely to become a useful adjunct to AST for the rapid identification of protein biomarkers and toxins of bacterial strains subjected to antibiotic stress.

A potential weakness of MS/MS-PSD for top-down proteomic identification is the mass/size of singly charged protein ions that can be fragmented. To date, the largest singly charged protein ion that fragments by PSD is around 16 kDa when ionized from an unfractionated bacterial cell lysate (data not shown). However, doubly and triply charged ions have shown increased fragmentation efficiency due to the Coulomb repulsion of their multiple charges. Although doubly and triply charged protein ions are less abundant than singly charged ions by MALDI, LC fractionation should enhance the absolute intensity of these multiply charged ions for MS/MS-PSD analysis.

Future directions

As MALDI-TOF and MALDI-TOF-TOF technology advances beyond pattern recognition analysis of unfractionated cell lysates or intact cells for bacterial identification, there will be an increasing impetus to identify specific protein ions whose amino-acid sequence and/or PTMs are unique to the source microorganism. Of particular interest is the identification of protein toxins and other virulence factors. One of the drivers of this impetus will be the expansion of genomic information available from whole genome sequencing using next generation sequencers. Transcriptomics, proteomics, and metabolomics are essential to provide information that is not available from a sequenced genome.

MALDI-TOF-TOF-MS/MS-PSD and CID (and other top-down proteomic techniques) are poised to make significant inroads into identification of nondigested proteins that have traditionally been dominated by other techniques, e.g., Edman sequencing, monoclonal antibodies (MAb), Western blots, ELISAs, etc. Although ISD (Calligaris et al. 2010) and "T^3-sequencing" (Suckau and Resemann 2003) will provide an alterative top-down MALDI-TOF-TOF approach, its use will be restricted to applications that can provide pure proteins and, thus, would exclude analysis of protein mixtures, such as unfractionated cell lysates or intact cells.

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References

Aebersold, R.; Mann, M. Mass spectrometry-based proteomics. *Nature* **2003**, *422*, 198–207.

Calligaris, D.; Villard, C.; Terras, L.; Braguer, D.; Verdier-Pinard, P.; Lafitte, D. MALDI in-source decay of high mass protein isoforms: application to alpha- and beta-tubulin variants. *Anal. Chem.* **2010**, *82*, 6176–6184.

Cook, A.; Reid-Smith, R. J.; Irwin, R. J.; McEwen, S. A.; Young, V.; Ribble, C. Antimicrobial resistance in *Campylobacter*, *Salmonella*, and *Escherichia coli* isolated from retail grain-fed veal meat from Southern Ontario, Canada. *J. Food Prot*. **2011**, *74*, 1245–1251.

Cotter, R. J.; Griffith, W.; Jelinek, C. Tandem time-of-flight (TOF/TOF) mass spectrometry and the curved-field reflectron. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci*. **2007**, *855*, 2–13.

Demirev, P. A.; Fenselau, C. Mass spectrometry for rapid characterization of microorganisms. *Annu. Rev. Anal. Chem*. **2008**, *1*, 71–93.

Demirev, P. A.; Feldman, A. B.; Kowalski, P.; Lin, J. S. Top-down proteomics for rapid identification of intact microorganisms. *Anal. Chem*. **2005**, *77*, 7455–7461.

Demirev, P. A.; Ho, Y. P.; Ryzhov, V.; Fenselau, C. Microorganism identification by mass spectrometry and protein database searches. *Anal. Chem*. **1999**, *71*, 2732–2738.

Durbin, K. R.; Tran, J. C.; Zamdborg, L.; Sweet, S. M.; Catherman, A. D.; Lee, J. E.; Li, M.; Kellie, J. F.; Kelleher, N. L. Intact mass detection, interpretation, and visualization to automate Top-down proteomics on a large scale. *Proteomics* **2010**, *10*, 3589–3597.

Fagerquist, C. K.; Sultan, O. Induction and identification of disulfide-intact and disulfide-reduced β-subunit of Shiga toxin 2 from *Escherichia coli* O157:H7 using MALDI-TOF-TOF-MS/MS and top-down proteomics. *Analyst* **2011**, *136*, 1739–1746.

Fagerquist, C. K.; Sultan, O. A new calibrant for matrix-assisted laser desorption/ionization time-of-flight-time-of-flight post-source decay tandem mass spectrometry of non-digested proteins for top-down proteomic analysis. *Rapid Commun. Mass Spectrom*. **2012**, *26*, 1241–1248.

Fagerquist, C. K.; Bates, A. H.; Heath, S.; King, B. C.; Garbus, B. R.; Harden, L. A.; Miller, W. G. Sub-speciating *Campylobacter jejuni* by proteomic analysis of its protein biomarkers and their post-translational modifications. *J. Proteome Res.* **2006**, *5*, 2527–2538.

Fagerquist, C. K.; Garbus, B. R.; Williams, K. E.; Bates, A. H.; Boyle, S.; Harden, L. A. Web-based software for rapid top-down proteomic identification of protein biomarkers, with implications for bacterial identification. *Appl. Environ. Microbiol*. **2009**, *75*, 4341–4353.

Fagerquist, C. K.; Garbus, B. R.; Miller, W. G.; Williams, K. E.; Yee, E.; Bates, A. H.; Boyle, S.; Harden, L. A.; Cooley, M. B.; Mandrell, R. E. Rapid identification of protein biomarkers of Escherichia coli O157:H7 by matrix-assisted laser desorption ionization-time-of-flight-time-of-flight mass spectrometry and top-down proteomics. *Anal. Chem*. **2010**, *82*, 2717–2725.

Fagerquist, C. K.; Sultan, O. Top-down proteomic identification of furin-cleaved α-subunit of Shiga toxin 2 from *Escherichia coli* O157:H7 using MALDI-TOF-TOF-MS/MS. *J. Biomed. Biotechnol*. **2010**, *2010*, 123460. doi: 10.1155/2010/123460.

Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. Electrospray ionization for mass spectrometry of large biomolecules. *Science* **1989**, *246*, 64–71.

Fenselau, C.; Demirev, P. A. Characterization of intact microorganisms by MALDI mass spectrometry. *Mass. Spectrom. Rev*. **2001**, *20*, 157–171.

Frankevich, V.; Zhang, J.; Dashtiev, M.; Zenobi, R. Production and fragmentation of multiply charged ions in "electron-free" matrix-assisted laser desorption/ionization. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 2343–2348.

Gonzalez, T.; Baudouy, J. Bacterial aminopeptidases: properties and functions. *J. FEMS Microbiol. Rev*. **1996**, *18*, 319–334.

Hardouin, J. Protein sequence information by matrix-assisted laser desorption/ionization in-source decay mass spectrometry. *Mass Spectrom. Rev.* **2007**, *26*, 672–682.

Hirel, P. H.; Schmitter, J. M.; Dessen, P.; Fayet, G.; Blanquet, S. Extent of N-terminal methionine excision from Escherichia coli proteins is governed by the side-chain length of the penultimate amino acid. *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 8247–8251.

Hu, Q.; Noll, R. J.; Li, H.; Makarov, A.; Hardman, M.; Graham Cooks, R. The orbitrap: a new mass spectrometer. *J. Mass Spectrom*. **2005**, *40*, 430–443.

Jarman, K. H.; Cebula, S. T.; Saenz, A. J.; Petersen, C. E.; Valentine, N. B.; Kingsley, M. T.; Wahl, K. L. An algorithm for automated bacterial identification using matrix-assisted laser desorption/ionization mass spectrometry. *Anal. Chem*. **2000**, *72*, 1217–1223.

Johannes, L.; Romer, W. Shiga toxins – from cell biology to biomedical applications. *Nat. Rev. Microbiol.* **2010**, *8*, 105–116.

Karas, M.; Bachmann, D.; Bahr, U.; Hillenkamp, F. Matrix-assisted ultraviolet laser desorption of non-volatile compounds. *Int. J. Mass Spectrom. Ion Processes* **1987**, *78*, 53–68.

Lay, J. O. Jr. MALDI-TOF mass spectrometry of bacteria. *Mass. Spectrom. Rev*. **2001**, *20*, 172–194.

Leduc, R. D.; Kelleher, N. L. Using ProSight PTM and related tools for targeted protein identification and characterization with high mass accuracy tandem MS data. *Curr. Protoc. Bioinform.* **2007** Sep; Chapter 13: Unit 13.6. doi: 10.1002/0471250953.bi1306s19.

Lin, M.; Campbell, J. M.; Mueller, D. R.; Wirth, U. Intact protein analysis by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom*. **2003**, *17*, 1809–1814.

Liu, Z.; Schey, K. L. Fragmentation of multiply-charged intact protein ions using MALDI-TOF-TOF mass spectrometry. *J. Am. Soc. Mass Spectrom*. **2008**, *19*, 231–238.

Mandrell, R. E.; Harden, L. A.; Bates, A.; Miller, W. G.; Haddon, W. F.; Fagerquist, C. K. Speciation of Campylobacter coli, C. jejuni, C. helveticus, C. lari, C. sputorum, and C. upsaliensis by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Appl. Environ. Microbiol*. **2005**, *71*, 6292–6307.

Medzihradszky, K. F.; Campbell, J. M.; Baldwin, M. A.; Falick, A. M.; Juhasz, P.; Vestal, M. L.; Burlingame, A. L. The characteristics of peptide collision-induced dissociation using a high-performance MALDI-TOF/TOF tandem mass spectrometer. *Anal. Chem*. **2000**, *72*, 552–558.

Ngan, G. J.; Lin, R. T.; Teo, J. W. Utility of the Bruker Biotyper matrix-assisted laser desorption ionisation time-of-flight mass spectrometer in a clinical microbiology laboratory. *Pathology* **2012**, *44*, 493–496.

Pineda, F. J.; Lin, J. S.; Fenselau, C.; Demirev, P. A. Testing the significance of microorganism identification by mass spectrometry and proteome database search. *Anal. Chem.* **2000**, *72*, 3739–3744.

Rajakaruna, L.; Hallas, G.; Molenaar, L.; Dare, D.; Sutton, H.; Encheva, V.; Culak, R.; Innes, I.; Ball, G.; Sefton, A. M.; Eydmann, M.; Kearns, A. M.; Shah, H. N. High throughput identification of clinical isolates of *Staphylococcus aureus* using MALDI-TOF-MS of intact cells. *Infect. Genet. Evol.* **2009**, *9*, 507–513.

Sandrin, T. R.; Goldstein, J. E.; Schumaker, S. MALDI TOF MS profiling of bacteria at the strain level: a review. *Mass. Spectrom. Rev.* **2012**, doi: 10.1002/mas.21359.

Solbiati, J.; Chapman-Smith, A.; Miller, J.; Miller, Ch.; Cronan, J. Jr. Processing of the N termini of nascent polypeptide chains requires deformylation prior to methionine removal. *J. Mol. Biol.* **1999**, *290*, 607–614.

Spengler, B. Post-source decay analysis in matrix-assisted laser desorption/ionization mass spectrometry of biomolecules. *J. Mass Spectrom.* **1997**, *32*, 1019–1036.

Stephan, R.; Cernela, N.; Ziegler, D.; Pflüger, V.; Tonolla, M.; Ravasi, D.; Fredriksson-Ahomaa, M.; Hächler, H. Rapid species specific identification and subtyping of *Yersinia enterocolitica* by MALDI-TOF mass spectrometry. *J. Microbiol. Methods* **2011**, *87*, 150–153.

Suckau, D.; Resemann, A. T3-sequencing: targeted characterization of the N- and C-termini of undigested proteins by mass spectrometry. *Anal. Chem.* **2003**, *75*, 5817–5824.

Suckau, D.; Resemann, A.; Schuerenberg, M.; Hufnagel, P.; Franzen, J.; Holle, A. A novel MALDI LIFT-TOF/TOF mass spectrometer for proteomics. *Anal. Bioanal. Chem.* **2003**, *376*, 952–965.

Syka, J. E.; Coon, J. J.; Schroeder, M. J.; Shabanowitz, J.; Hunt, D. F. Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 9528–9533.

Tanaka, K.; Ido, Y.; Akita, S.; Yoshida, Y.; Yoshida, T. Detection of high mass molecules by laser desorption time-of-flight mass spectrometry. *Proceedings of the 2nd Japan-China Joint Symposium on Mass Spectrometry*, Osaka, Japan, Abstract, 1987, pp. 185–188.

van Belkum, A.; Welker, M.; Erhard, M.; Chatellier, S. Biomedical mass spectrometry in today's and tomorrow's clinical microbiology laboratories. *J. Clin. Microbiol.* **2012**, *50*, 1513–1517.

Wang, M.; Marshall, A. G. A "screened" electrostatic ion trap for enhanced mass resolution, mass accuracy, reproducibility, and upper mass limit in Fourier transform ion cyclotron resonance mass spectrometry. *Anal. Chem.* **1989**, *61*, 1288–1293.

Washburn, M. P.; Wolters, D.; Yates, J. R. 3rd. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat. Biotechnol.* **2001**, *19*, 242–247.

Williams, T. L.; Leopold, P.; Musser, S. Automated post-processing of electrospray LC/MS data for profiling protein expression in bacteria. *Anal. Chem.* **2002**, *74*, 5807–5813.

Zhang, L.; Reilly, J. P. Peptide *de novo* sequencing using 157 nm photodissociation in a tandem time-of-flight mass spectrometer. *Anal. Chem.* **2010**, *82*, 898–908.

Zubarev, R. A.; Kelleher, N. L.; McLafferty, F. W. Electron capture dissociation of multiply charged protein cations. A nonergodic process. *J. Am. Chem. Soc.* **1998**, *120*, 3265–3266.

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