Chapter 2
Methods and Instrumentation in Mass Spectrometry for the Differentiation of Closely Related Microorganisms

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Introduction

Mass spectrometer instruments can be considered as a complex chemical reaction vessel, and as such, the resulting mass spectrum (i.e., the "product" of these reactions) is directly related to all experimental parameters, including, but not limited to, sample preparation, instrument settings, and environmental conditions. Because of its highly informative data output, mass spectrometry (MS) has found many applications in the analysis and quantitation of small to large molecular weight (MW) compounds in areas of energy, environment, forensics, space exploration, and in clinical and biological laboratories, to name just a few. To this list of applications, the analysis of microorganisms has proven to be an accurate and cost-effective approach in clinical settings. Because microorganisms can be considered as a complex chemical sample, its preparation is closely related to the information being sought, and this in turn will determine the type of MS instrumentation to be used. Unfortunately, a single sample preparation protocol will not provide a compatible sample state for all types of mass spectrometers (and vice versa). This relationship between methodology and instrumentation is illustrated (albeit simplified) in Fig. 2.1, where the final sample state prior to analysis is matched with the type of sample preparation required, instrumentation(s), and required data processing.

This relationship between the final state of the sample and MS instrumentation is mainly a consequence of the type of sample inlet and ionization technique used in a particular mass spectrometer. Referring to Fig. 2.1, the analysis of intact cells by matrix-assisted laser desorption/ionization-MS (MALDI-MS), (Jaskolla and Karas 2011) one of the simplest approaches for microorganism analysis by MS, (Holland et al. 1996) requires the isolation of a pure microbial colony, which is then deposited directly onto the MALDI plate. The subsequent mass spectral profiles,

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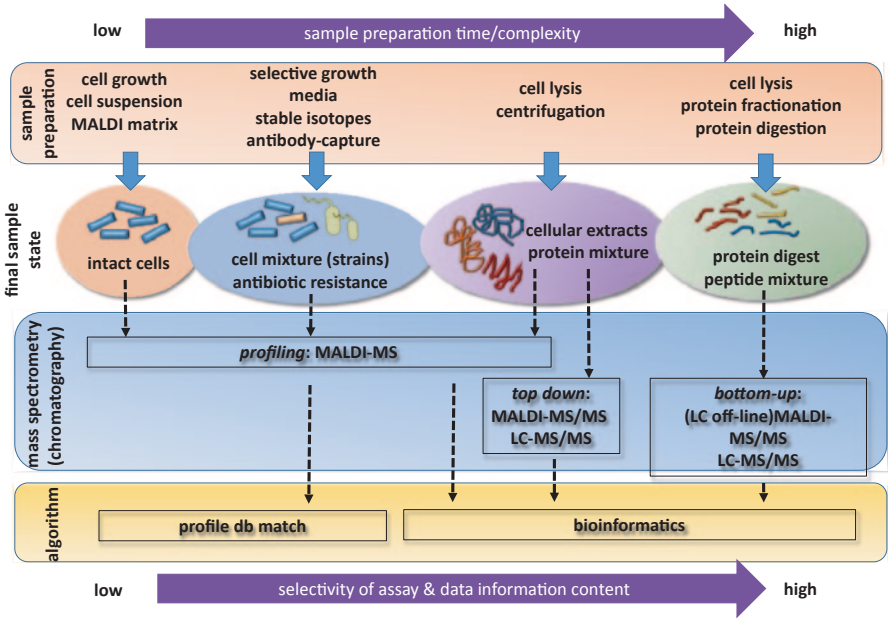

Fig. 2.1 Relationship between sample preparation time and complexity for several MS-based methods for the analysis of microorganisms

consisting mostly of ribosomal proteins, (Holland et al. 1999; Ryzhov and Fenselau 2001) are then used to classify, differentiate, and identify the microorganism. This approach requires the use of standard mass spectral databases of known microorganisms that have been acquired using the same experimental conditions. If on the other hand, one does not possess such standard mass spectral databases, a bioinformatics approach can be used. In one approach requiring a pure microbial sample, the experimentally obtained protein masses are matched to a proteome database (Demirev et al. 2001). In a second approach, a protein signal is selected for gas-phase fragmentation (tandem MS or MS/MS) and the observed ions in the tandem mass spectrum are then matched to expected fragmentation patterns of proteins contained in a proteome database (Fagerquist et al. 2010). This top-down proteomics approach can also be enhanced (i.e., more proteins detected) by the use of a liquid chromatography (LC) separation/fractionation step followed by MS/MS of the intact protein ions (McFarland et al. 2014). However, this enhancement in selectivity comes with additional sample preparation steps to extract proteins and remove other cell components incompatible with the LC step. Because of the unique ion chemistry of the protein fragmentation process and the large mass-to-charge ratios (m/z's) of the resulting fragment ions, these top-down analyses require the use of specialized MS instrumentation that allow for the fragmentation of large protein ions and the analysis of their fragment ions with sufficient mass accuracy to provide meaningful database search results. Lastly, this bioinformatics approach can be per-

formed in a bottom-up mode where the sample preparation includes protein extraction followed by site-specific enzymatic digestion (e.g., with trypsin). The resulting complex mixture of peptides is analyzed by LC-MS/MS, and the acquired tandem mass spectrum for each peptide is matched, via a database search, to the protein originating the peptide, and if possible, its biological origin (i.e., the microorganism). The increased level of complexity for the sample preparation and/or analysis steps for both top-down and bottom-up proteomic approaches results in the highest degree of selectivity of all MS-based methods as a mixture of microorganisms can, in principle, be identified, regardless of growth conditions. Finally, the ability of MS to detect isotopologues allows the use of stable isotopes (e.g., ^{13}C, ^{15}N, or ^{18}O) to differentiate and/or quantitate biomolecules between two different cell states, as in the detection of antibiotic-resistant strains of microorganisms.

The following discussions will focus on factors affecting the ability of MS-based methods to achieve high levels of specificity and selectivity that are required in the detection of closely related bacteria and the detection of antibiotic-resistant strains, followed by a description of MS instrumentation, and examples from the current scientific literature.

Selectivity and Specificity in the Analysis of Microorganisms with MS

In the differentiation or identification of microorganisms, several factors are influential in determining the *specificity of a technique for* a target microorganism or the ability of a technique to *select among* several closely related microorganisms (i.e., selectivity). General strategies to achieve these goals include:

1. *Increasing the selectivity of the measurement to differentiate among unique features that define a certain microorganism*. This strategy may include the addition of a chromatographic step and/or increasing the mass resolution and mass accuracy of the mass spectrometer (time-of-flight (TOF), Fourier transform (FT) orbitrap or FT-ion cyclotron resonance (ICR) mass analyzers).
2. *Decreasing the overall variance of the measurement in order to detect subtle differences in traits common to all samples*. In this instance, the goal is to detect subtle differences in the pattern between two mass spectra, each obtained from different species and/or strain. Thus, the differentiation of two closely related microorganisms depends on the quantitative (relative) detection of small differences in signal strength common to both samples. Factors affecting the overall measurement variance (s^2, where s is the standard deviation) are additive and ideally independent of each other, with the total variance of an analysis being the sum of the individual steps in the analysis

$$s^2_{analysis} = \sum_{i=1}^{n} s_i^2,$$

where *i* is the individual step (e.g., sampling, sample preparation, measurement, data processing) in the overall analysis. In general, it has been recognized that the individual variances in the analysis follow the trend:

$$s^2(\text{sampling}) > s^2(\text{sample prep}) >> s^2(\text{measurement})$$

Therefore, it is usually the case for most analytical protocols to focus on decreasing the variance contributions of the sampling and sample preparation steps. Manufacturers of modern chemical instrumentation, with the availability of advanced electronic components and signal processing, have considerably decreased the contribution of the measurement to the overall analysis variance. The use of automation in both sample preparation and data acquisition is key in a strategy to reduce the overall variance of the analysis. The contribution due to sampling can be reduced by increasing the number of biological samples analyzed (replicate samples).

3. *Increasing the specificity of the measurement for a target microorganism.* Factors that may increase the specificity for a target microorganism include the incorporation of a selective growth media step (antibiotic resistant), DNA amplification, antibody capture/enrichment, stable-isotope labeling, and multistage mass analyses (e.g., tandem MS or MS/MS, selective reaction monitoring or SRM, vide infra).

Approaches involving these strategies will be addressed in subsequent sections of this chapter with examples from the recent literature. However, a brief review of the MS instrumentation involved in these measurements will be presented first.

MS Instrumentation

The analysis of microorganisms with MS-based techniques involves a wide range of instrumentation, and knowledge of their capabilities and limitations is key in extracting the most information from the analysis. Two components are fundamental in defining the capabilities of any MS instrument and include the type of (i) ionization and (ii) mass analyzer used. For the techniques relevant to the characterization of biomarkers in microorganisms being discussed here, only MALDI and electrospray ionization (ESI), with the TOF, quadrupole(s), and orbitrap mass analyzers, will be described in detail. However, regardless of the type of MS instrument being used, a common operational requirement is that the final state of the sample, prior to mass analysis, be gas-phase ions of either positive or negative polarity. These gas-phase ions are then separated or sorted based on their mass-to-charge ratio or *m*/*z,* a dimensionless quantity (Price 1991; Gross 2011). (For convenience, mass, *m,* is expressed in terms of the unified atomic mass unit, which is defined as 1/12 the mass in kilograms of one atom of ^{12}C, u or $m_u = 1.66054 \times 10^{-27}$ kg. Thus, the quantity *m* is the ratio of the mass in kilograms of the ionized molecule divided by m_u or

$m = m(kg)/m_u(kg)$. The quantity z represents the number of elementary charges on the ion, which is also a dimensionless number) (Boyd 2008). All mass analyzers are operated under vacuum ($\sim 10^{-4}$–10^{-12} Torr), their magnitude depends on the mode of operation, and are required in order to avoid collisions of the analyte gas-phase ion with neutral molecules present in air (as well as avoiding arcing within components in the mass analyzer held at high voltages). This increases signal sensitivity and avoids unwanted ion–molecule reactions between the analyte ion and reactive gaseous species (e.g., oxygen).

Both MALDI and ESI are unique in their ability to form gas-phase ions from large MW molecules, biological or synthetic, without inducing fragmentations, and are thus considered to be "soft" ionization techniques (unlike "hard" ionization techniques like electron ionization (EI) which induce fragmentations *during* the ionization step) (McLafferty and Tureek 1993). ESI is considered an atmospheric pressure (AP) ionization technique since ions are generated outside the mass analyzer vacuum manifold. Although MALDI is usually conducted under vacuum in TOF-MS instruments used for bacteria identification, MALDI can also be performed under AP conditions, (Laiko et al. 2000; Madonna et al. 2003) allowing its use with instruments originally setup to use ESI, like the triple quadrupole MS.

MALDI and MALDI-MS Instrumentation

The development of MALDI by Hillenkamp and coworkers (Karas and Hillenkamp 1988) allowed for the analysis of high MW biological (e.g., proteins) and synthetic (e.g., polymers) samples without inducing fragmentation. The MALDI process relies on mixing an organic compound, termed the matrix, with the biological sample, the former in a 100:1 to 1000:1 molar excess. When the mixture is dried, the organic compound forms a heterogeneous crystalline matrix (Fig. 2.2) that surrounds and isolates individual analyte molecules in the original biological sample. Upon irradiation by a pulsed laser (UV laser in most commercial instruments), the photon energy is absorbed predominantly by the matrix compound and this electronic excitation is converted into thermal (vibrational) and translational energy, ablating (i.e., desorbing) matrix molecules as well as intact and ionized analyte molecules into the gas phase (Zenobi and Knochenmuss 1998). As such, the MALDI process is considered a pulsed ion source as it generates discrete packets of ions.

Because of the heterogeneous nature of the MALDI matrix when dry, ion yields at different locations within a MALDI matrix are not the same, leading to the description of these locations within the sample as "hot" or "cold" spots to refer to locations yielding intense or weak signals, respectively. The presence of these hot and cold signal spots within the MALDI matrix limits the usefulness of the MALDI process as a quantitative tool, imposing the need to acquire, on average, several hundred mass spectra from different locations within a sample in order to obtain a representative (average) mass spectrum.

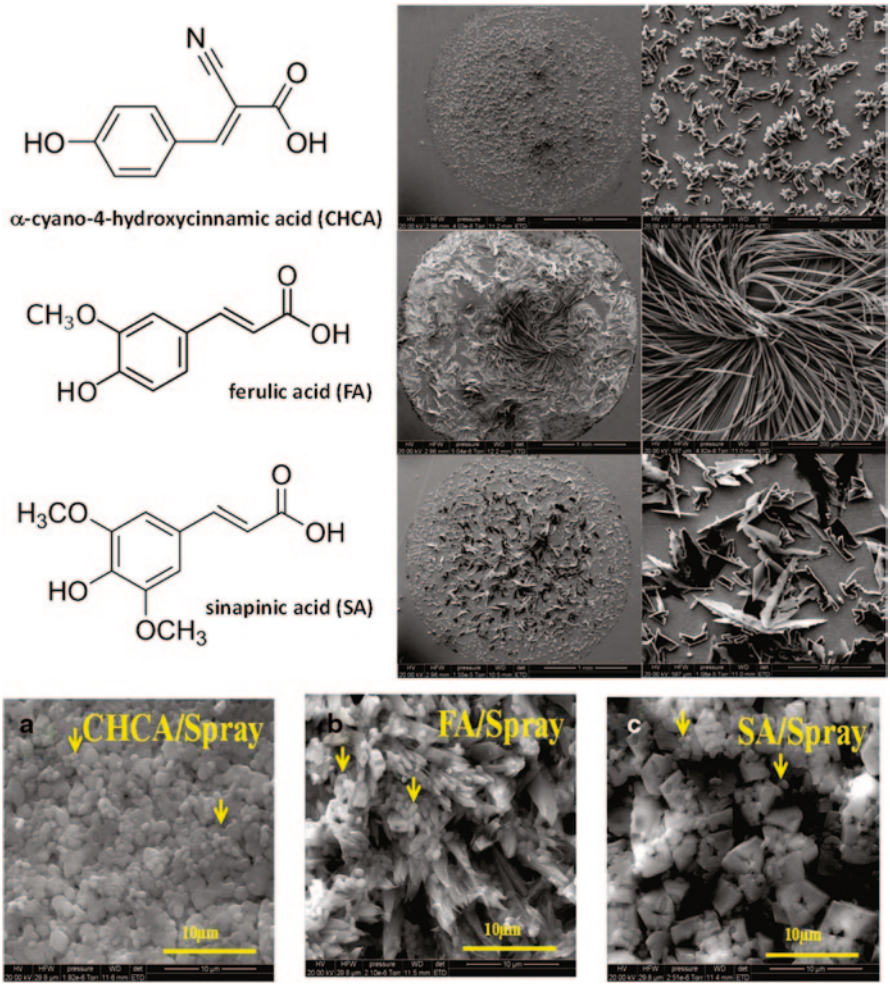

Fig. 2.2 Structures and scanning electron microscopy *(SEM)* photographs of different MALDI matrices deposited onto a stainless steel plate. *Lower* SEM photographs show *E. coli* cells co-crystallized with different MALDI matrices (matrix applied with a spray deposition technique). *Arrows point* to intact cells within the crystalline matrix. (Adapted from Toh-Boyo et al. 2012, copyright American Chemical Society)

The MALDI ionization process is very complex and depends heavily on the type of analyte molecule, matrix used, and laser fluence, but a recent study (Jaskolla and Karas 2011) suggests that two ionization models are mainly at play: (1) charge separation during the desorption step of *preformed ions* embedded in the crystalline matrix (a.k.a., the "Lucky Survivor" model), and (2) gas-phase protonation via ion–molecule reactions during the desorption step. In the analysis of biological molecules, the MALDI process yields primarily single-charged ions, either due to protonation or cation adduct formation (e.g., $[M+H]^+$ or $[M+Na]^+$, where M is the neutral molecule) in positive ion mode or deprotonation in negative ion mode (e.g.,

$[M-H]^-$). This fact is particularly useful when analyzing a mixture of proteins as it yields a simplified mass spectrum without overlapping signals. Important to note when analyzing complex mixtures of biomolecules with MALDI is the signal suppression effect, which takes place during the ionization process. For example, in positive ion mode the signal from a highly abundant, but acidic protein may be suppressed by the presence of a low abundant, but basic protein which yields an intense signal. As a result, what is seen in the mass spectrum is neither a quantitative nor a qualitative reflection of the composition of the sample. This effect is clearly exemplified in the MALDI-MS analysis of intact bacterial cells, which mostly yields signals due to ribosomal proteins while DNA, metabolites, lipids and other high MW proteins remain undetected.

Part of the success of MALDI-MS for the analysis of microorganisms derives from the simplicity and robustness of the methodology, and in its simplest form, intact or whole cells can be deposited directly onto the MALDI plate or mixed with the matrix solution and analyzed directly. Many methods have been published describing this process, but it is believed that bacterial cells are lysed and proteins extracted into the matrix solution in the minutes before crystallization (i.e., during solvent evaporation on the plate, ~1–2 min), even though preserved cell integrity has been observed in microphotographs of the co-crystallized bacteria-matrix sample (Fig. 2.2) (Toh-Boyo et al. 2012; Madonna et al. 2000). This is backed by the fact that protocols using either solvent extraction or intact cells are both effective in producing similar protein signals, albeit with different profiles (i.e., relative peak intensities) (Basile 2011).

As mentioned earlier, it is generally agreed that the majority of the proteins observed in the analysis of bacterial cells with MALDI-MS are ribosomal proteins in the molecular mass range of 2–20 kDa (Holland et al. 1996, 1999; McFarland et al. 2014; Suarez et al. 2013). This is the case since they are abundant (almost half of the mass of growing cells), basic ($pI > 9$, easily ionized under mild acid conditions), and slightly hydrophilic in nature (easily solubilized when mixed with the matrix solution) (Ryzhov and Fenselau 2001). These facts highlight the importance of solvent composition and control of every step (i.e., exact sequence of events) (Cohen and Chait 1996) in the sample preparation protocol for MALDI-MS of bacteria, as they dictate the range of proteins detected, their observed signal strength, and overall signal pattern.

TOF Mass Analyzer

The TOF mass analyzer is suitable to measure the m/z distribution of discrete pulsed ion sources, unlike a continuous stream of ions, and for this reason it is usually coupled with MALDI, a pulsed ion source. In a TOF-MS, a discrete packet of ions with different m/z's (generated via MALDI) are accelerated to the same kinetic energy by applying a voltage (U~10–25 kV; direct current, DC) to the stainless steel sample plate. These ions enter a field-free region (no voltage or magnetic fields

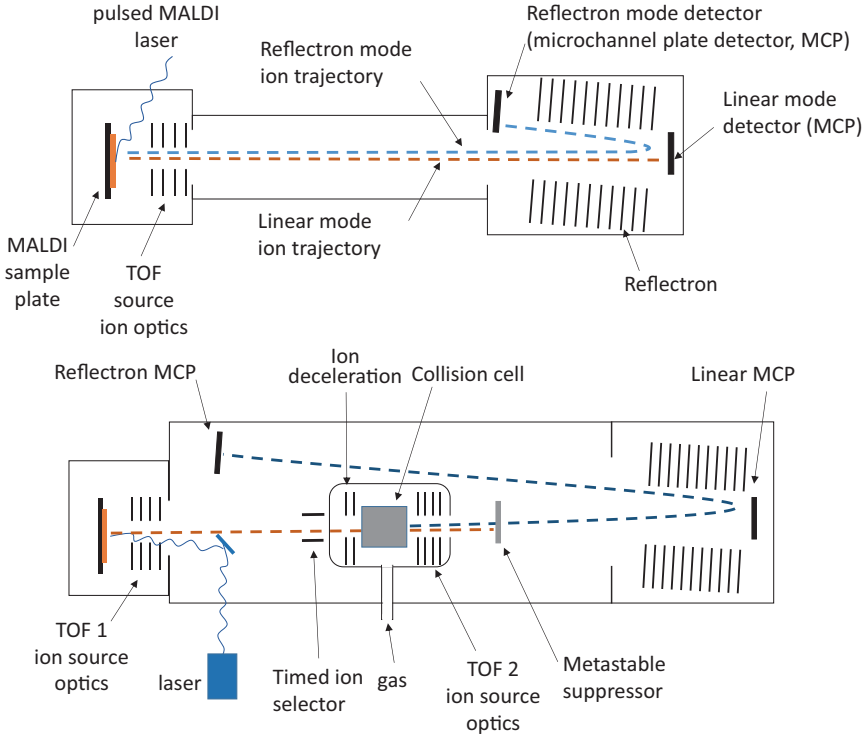

Fig. 2.3 Simplified general diagrams of **a** MALDI-TOF-MS and **b** MALDI-TOF/TOF-MS (based on the Sciex 4800/5800™ systems)

applied) where ions with small *m*/*z*'s travel faster than those with large *m*/*z*'s, and the different times to travel a predefined distance *(d)* forms the basis for their mass separation. The simplified relationship between TOF (t_{TOF}) and *m*/*z* is given by:

$$t_{TOF} = \frac{d}{\sqrt{2U}}\sqrt{\frac{m}{z}}.$$

In principle, the TOF-MS does not have an upper mass limit; however, in practice they are limited by the efficiency of the multichannel plate (MCP) detector (vide infra, Fig. 2.3) in converting low kinetic energy ions (i.e., large *m*/*z*'s) into a detectable electrical current, and the ability of the ionization source to produce ions of large *m*/*z*. Operationally, the relationship between *m*/*z* and t_{TOF} is established by calibration with a set of standard compounds of known *m*/*z* values for their $[M+H]^+$ ions. This calibration is dependent on matrix type and laser intensity (each affects the initial ion velocity during desorption) and the sample position within the MALDI plate (affecting the distance traveled, *d,* and the effective accelerating voltage, *U,* experienced by the desorbed ion). A simplified general diagram of a MALDI-TOF-MS instrument is illustrated in Fig. 2.3a.

Instruments based on this design (linear, but not necessarily reflectron) form part of most, if not all, of the commercially available MALDI-TOF-MS microorganism identification systems that are based on matching a mass spectrum to a mass spectral library of microorganisms (i.e., profile-based MALDI-MS). When operated in the reflectron mode to increase the mass resolution of the measurement (practical up to ~ *m*/*z* 5000), current state-of-the-art TOF mass analyzers (with a properly designed MALDI ion source) specify mass accuracies in the 1 ppm or ± 0.001 (at *m*/*z* 1000). However, no peptide/protein sequence information can be derived from this mass measurement alone and an additional level of selectivity, tandem MS or MS/MS, is required to obtain this information.

Another type of MS available with the MALDI ion source and based on the TOF mass analyzer is the tandem TOF or TOF/TOF-MS. This configuration has two TOF mass analyzers configured in series, separated by a collision cell. This configuration has the capability of obtaining mass spectral protein profiles as well as sequence information of peptides (up to ~4000 Da), and for one manufacturer mid-sized proteins (5–15 kDa). A simplified schematic of a MALDI-TOF/TOF-MS instrument is illustrated in Fig. 2.3b (the following discussion is based on the Sciex 4800™ system (Yergey 2002). An excellent discussion of the inner workings of the Bruker MALDI-TOF/TOF-MS system can be found in Suckau et al. (2003)). Ions formed in the MALDI ion source are accelerated toward the first TOF mass analyzer (TOF1), where ions are separated according to their *m*/*z*'s. In the MS mode, ions are allowed to travel uninterrupted to either the linear or reflectron detector. In the tandem MS (MS/MS) mode, ions of a single *m*/*z* value are selected and allowed to enter the collision cell. This *m*/*z* selection is performed via a timed ion selector, with a series of voltages applied at a unique time on the path of the ion beam so as to deflect all ions except those of the desired *m*/*z* (or TOF). The selected ion is introduced into the collision cell filled with a neutral gas like argon or nitrogen, and upon collision, fragment ions are formed via collision-induced dissociation (CID). Precursor ions can also undergo fragmentation during the MALDI process, via laser-induced dissociation (LID), (Suckau et al. 2003) or after the MALDI process, via post-source decay (PSD), (Neubert et al. 2004; Fagerquist 2013) where metastable ions leave the MALDI ion source and fragment during their voyage through TOF1. In all these fragmentation events, CID, LID, or PSD, the generated fragment ions will have roughly the same velocity as the precursor ion, and thus they cannot be discriminated by their *m*/*z*'s within the TOF1 mass analyzer. The TOF/TOF instrument achieves mass separation of these fragment ions (and obtains useful sequence information) by re-acceleration of this ion packet into the second TOF mass analyzer (TOF2). This second acceleration event becomes the starting point for recording the fragment ion mass spectrum (Yergey et al. 2002). For small proteins (15 kDa), this type of instrument can be used for top-down proteomic measurements, where the fragmentation of a single protein signal from a mixture can yield sequence information about the precursor ion and has been used to discriminate proteins varying by a single amino acid in their sequence (Fagerquist et al. 2010). Finally, when coupled with offline LC and fraction collection directly onto the MALDI plate, this type of instrumentation allows for bottom-up proteomic measurements (Marcus et al. 2007; Benkali et al. 2008; Bodnar et al. 2003).

ESI and ESI-MS Instrumentation

The development of ESI as an ionization source for MS by Yamashita and Fenn (1984) allowed the formation of gas-phase ions of biomolecules in liquid samples, thus enabling the analysis of intact proteins in solutions and of samples separated by LC. A detailed discussion of ESI is beyond the scope of this chapter as many excellent reviews and books have been written on the subject(Bruins et al. 1998; Cech and Enke 2001; Cole 2008). In general, the ESI process in positive ion mode for most biomolecules (proteins and peptides) starts in an acidified solution, that is, by the formation of ions via protonation of basic groups. This is typically accomplished by the addition of a volatile organic acid like acetic acid or formic acid (1 % or 0.1 %, respectively) in a 50 % organic-aqueous solvent (methanol or acetonitrile). The solution is then driven into a metal capillary (~ 50–100 μm inner diameter) connected to a power supply at 3–4 kV (DC voltage). As the liquid emerges at the open end of the capillary, the large electric field causes charge separation of the preformed ions in solution. In the case of biomolecules, an ionized peptide (positive charge) is separated from either a formate ion ($HCOO^-$) or the acetate ion (CH_3COO^-). The use of trifluoroacetic acid (TFA) to acidify solutions for ESI analysis is discouraged as the CF_3COO^- ion forms a strong ion pair with the positively charged biomolecule, making charge separation difficult and thus lowering the ionization efficiency of the ESI process. This accumulation of positively charged ions at the open end of the capillary causes the deformation of the liquid meniscus into what is termed a Taylor cone. Eventually, the electrostatic repulsive forces between the positive charges accumulated in the meniscus exceed the surface tension of the liquid leading to the formation of a fine jet of liquid, which breaks into fine droplets, each containing an excess of positively charged molecules. According to the ion evaporation model (IEM), (Nguyen and Fenn 2007) these droplets undergo a cascade of evaporative and Coulomb fission (charge repulsion) cycles until droplets of about 10 nm in diameter are formed. At this droplet size the effective electric field at the surface is large enough to push one or more solvated ions into the gas phase. A second ionization model, the charge residue model (CRM), describes the generation of an ion when all the solvent is evaporated from the droplet. Although there are many studies showing the prevalence of one model over the other in ESI, the consensus is that large ionized molecules (1000 u) are generated by a process closely described by the CRM. On the other hand, smaller and solvated ions can be emitted from nano-droplets by a process better described by the IEM (Wilm 2011).

In general, for positive ion mode ESI, ionization efficiency is dictated not only by the basicity of the molecule, but also by its hydrophobicity, which determines its concentration at the surface of the droplet (i.e., surface activity). As a result, not all biomolecules present in the sample are ionized with the same efficiency. That is, basic and hydrophobic molecules (with a high surface activity) tend to ionize more efficiently than basic and highly polar molecules. For example, a peptide with a high content of hydrophobic amino acids (phenylalanine, tryptophan) will experience a higher ESI ionization efficiency than a peptide of the same charge but with amino

acids with polar side chains (serine, aspartic acid). Therefore, in the ESI-MS analysis of a complex mixture (e.g., a protein mixture derived from bacteria cell lysate), the observed mass spectrum is neither a quantitative nor qualitative reflection of the composition of the sample. Molecules in the sample with high hydrophobicity and basicity will ionize more efficiently than molecules with lower hydrophobicity and/or basicity, even though the latter may be present at higher concentration. Another characteristic of ESI is the fact that proteins are ionized at multiple sites yielding charge state distributions of ions with multiple protons: $[M+H]^+$, $[M+2H]^{2+}$, …, $[M+nH]^{n+}$. In general, ionization suppression effects in ESI are more pronounced than in MALDI, and coupled with the possibility of observing overlapping charge state distribution from different proteins, ESI-MS is not as straightforward as MALDI-MS for the analysis of complex protein mixtures and is the main reason that ESI-MS is usually coupled with online LC separation.

However, LC-(ESI)-MS offers several key advantageous features for the analysis of closely related bacteria. First, the analysis of large proteins, above 20 kDa, is possible by LC-ESI-MS, increasing the dynamic range of biomarkers available for detection (Everley et al. 2008). In addition, the CID process is more efficient when performed on ions with large charge states (Schaaff et al. 2000) (i.e., large z values), resulting in information-rich fragmentation mass spectra that can identify the precursor peptide (bottom-up proteomic) or protein (top-down proteomic) by its unique amino acid sequence (McFarland et al. 2014). This process, however, requires time-consuming sample preparation and far more complex instrumentation and data analysis than the whole-cell bacteria-MALDI-MS approach (see Fig. 2.1).

Quadrupole-Based Mass Analyzers

One of the earliest MS instruments to be interfaced with ESI is quadrupole-based mass analyzer (Fenn et al. 1989). Early work on the use of quadrupole-based MS instruments for the analysis of microorganisms focused on the detection of mostly lipid biomarkers like phospholipids, triglycerides, and free fatty acids (Anhalt and Fenselau 1975; Meuzelaar and Kistemaker 1973; Huff et al. 1986; Goodacre et al. 1998; Boon et al. 1981; Guckert et al. 1986; DeLuca et al. 1992). In addition to using targeted extraction/derivatization protocols, these early investigations also incorporated rapid thermal desorption and/or pyrolysis methods (with EI) to directly analyze intact bacteria in a manner of minutes (DeLuca et al. 1990). More recently, the triple quadrupole MS (QQQ; or QqQ, where q signifies the collision cell, Q2; vide infra) (Fig. 2.4a) in conjunction with ESI has been used for highly specific detection of microorganisms via targeted bottom-up proteomic approaches (Karlsson et al. 2012; Picotti and Aebersold 2012).

The quadrupole mass analyzer is truly a scanning instrument, in that only ions of a particular m/z can be transmitted through the device (i.e., have a stable trajectory) at a particular time, and thus it is often referred to as a mass filter. The quadrupole mass analyzer consists of a set of four metal rods, ideally each having a parabolic

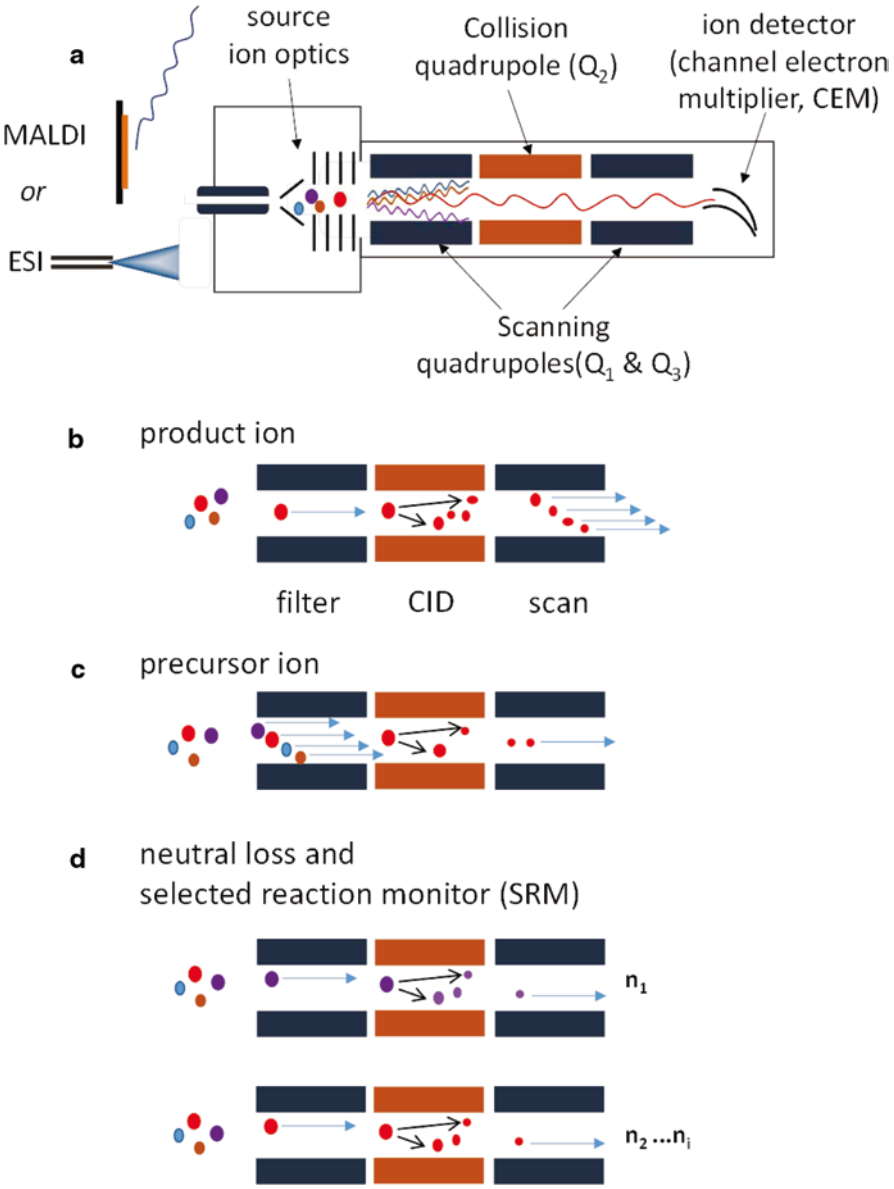

Fig. 2.4 Triple quadrupole MS, QQQ, where Q1 and Q3 indicate scanning quadrupoles and Q2 indicates the collision cell consisting of either an RF-only quadrupole, hexapole, or octapole. In some hybrid instruments Q3 is replaced by either a TOF or a linear quadrupole ion trap. (See text for more details and references for further reading)

surface shape, connected to a DC and radio-frequency (RF) power supplies. By varying the DC and RF voltages applied to opposite rods in the quadrupole mass analyzer (while maintaining the ratio of their magnitudes constant), the stability of ions with different *m*/*z* through the device is sequentially varied, effectively scanning a user-selected and predefined *m*/*z* range. Because modern electronics are able to control voltages and frequencies with high accuracy and precision, these instruments are well suited for quantitative measurements. In addition, the ability of these instruments to perform tandem-MS (MS/MS) measurements makes them ideal for the design of highly specific methodology for the detection of a wide range of chemical and biological species.

The QQQ-MS can be operated in four main scan modes: (i) full scan, (ii) product ion scan, (iii) precursor ion scan, and (iv) SRM (Fig. 2.4a–d). In the full-scan mode, usually Q1 is scanned, while both Q2 and Q3 are operated in RF mode only, essentially acting as ion guides (Fig. 2.4a). In the product ion mode (Fig. 2.4b), Q1 is set to pass only ions of a single *m*/*z,* filtering out all other ions formed in the ion source, and the collision cell, Q2, is operated in RF mode and filled with argon gas ($0.5–2 \times 10^{-3}$ Torr or ~0.2 Pa). Preselected ions emerging from Q1 are accelerated into Q2 where they undergo inelastic collisions with the argon gas, inducing molecular ion dissociation that yields both neutral fragments (not detected) and fragment ions. These fragment ions are then sorted according to their *m*/*z*'s by the scanning Q3. The precursor ion scan mode (Fig. 2.4c) is useful in situations where there is a need to determine the source (precursor) of a particular fragment ion or to survey members of a particular class of compounds that have a common fragment ion (e.g., glycerophospholipids produce a common fragment ion at *m*/*z* 184 regardless of the mass of the precursor ion) (Murphy et al. 2001). To accomplish this measurement, Q1 is scanned and a narrow *m*/*z* window of ions is sequentially introduced into Q2 and fragmented, while Q3 is set fixed at the particular *m*/*z* value of the (common) fragment ion. The mass spectrum is then plotted with the intensity of the fragment ion versus the mass scale of Q1 (not Q3 since it is fixed at a single *m*/*z*). The SRM mode is a highly specific mode of operation of the QQQ instrument where the specific precursor–fragment ion relationship is measured (Fig. 2.4d). For example, a particular peptide known to be a specific biomarker for a disease or microorganism can be detected by setting Q1 to its precursor *m*/*z* value and Q3 to a unique fragment ion of this precursor. The precursor ion, upon fragmentation in Q2 will produce the specific fragment ion that will be transmitted through Q3 and detected. The specificity of the assay is directly related to the specificity of the precursor-product transition (i.e., of the fragmentation reaction), and thus many validation measurements must be performed prior to SRM measurements (Picotti and Aebersold 2012; Lange et al. 2008). In practice, several of these precursor-product reactions can be measured sequentially, and thus the term multiple reaction monitoring (MRM) is also used.

In some hybrid triple quadrupole-based MS systems, the last quadrupole (Q3) is replaced with a TOF analyzer, with an ion path set at a 90° angle from the ions exiting the second quadrupole (or collision cell), and thus it is often termed an

orthogonal TOF. This quadrupole-TOF setup, or Q-TOF (or Qq-TOF, where q signifies the collision cell) increases the resolution and mass accuracy of the product mass spectrum and is also used for bottom-up proteomic measurements (Martinez et al. 2010; Mott et al. 2010; Alvarez et al. 2013). In another hybrid configuration, Q3 is replaced by a linear quadrupole ion trap mass analyzer, which allows ion accumulation for increased sensitivity and MS^n capability (Londry and Hager 2003).

Orbitrap Mass Analyzer

The orbitrap mass analyzer is an ion trap device that provides high accuracy and resolution mass measurement without the need of a magnetic field, (Hu et al. 2005; Zubarev and Makarov 2013) and thus it is more accessible in terms of lab requirements, and initial and operating costs. Some consider it to be the "gold standard" mass spectrometer for proteomic-based measurements (Mitchell 2010). The orbitrap mass analyzer is usually found in a hybrid configuration interfaced with a linear ion trap mass analyzer and transfer octopoles and C-trap (Fig. 2.5) (Senko et al. 2013).

In its core operation mode, ions injected into the orbitrap are trapped in an electrostatic field and oscillate along the central electrode (*z*-axis) with a periodic back

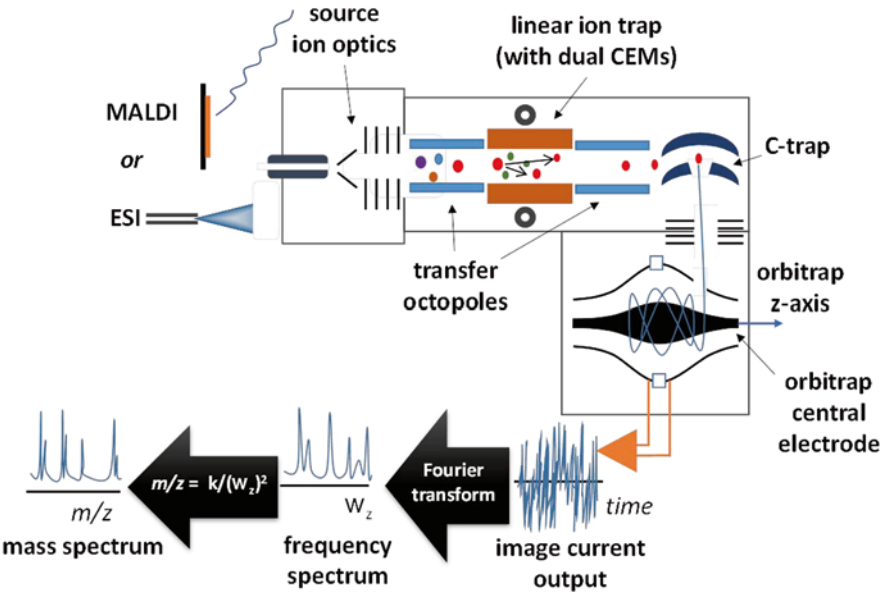

Fig. 2.5 Diagram of a hybrid linear ion trap and orbitrap mass spectrometer

and forth motion. The frequency of this axial oscillation (w_z) is inversely proportional to the square root of the m/z of the trapped ions.

$$\omega_z = \sqrt{k\frac{q}{m}},$$

where the electric charge q is equal to multiples (z) of the electron charge (e) and k is a parameter describing the field (Makarov 2000). The ion oscillations are recorded in the time domain by detecting the transient image current on the electrodes. The respective ion frequencies are derived from the transient by a fast FT and the corresponding m/z values are determined using the above equation.

Orbitrap-based MS systems are predominantly used for bottom-up and middle-down proteomic measurements, (Cannon et al. 2010) where the peptides are fragmented in the linear ion trap and fragments mass analyzed in the orbitrap. In addition, orbitrap-MS systems are used for accurate mass determination of intact proteins (McFarland et al. 2014). These measurements are particularly useful for the detection and differentiation of closely related microorganisms since this methodology allows the detection of a wide range of proteins in the sample, beyond the detection of ribosomal proteins.

MS-Based Methods and Instrumentation for the Differentiation of Closely Related Bacteria: Strain Level and Antibiotic Resistant

The analysis of microorganisms by MALDI-MS has been successful since the range of biomarkers detected afford the required level of selectivity to differentiate samples among a wide range of microorganisms, bacteria and fungi included, thus allowing nontargeted analyses. Extensive work published in the literature demonstrates the ability to obtain phylogenetic classification via MALDI-MS, equivalent to that obtained by 16S rRNA (Seng et al. 2009; Hsieh et al. 2008; Boehme et al. 2013; Li et al. 2014; Deng et al. 2014). It is natural that the use of MALDI-MS has been extended to the identification of bacteria at the strain level and for the differentiation of antibiotic-resistant strains. In this section, the MS-based techniques used in achieving these goals will be highlighted with selected examples from the literature, with an emphasis on MS-hardware and technique (i.e., sample preparation). The reader is also referred to more comprehensive literature reviews on the detection of microorganisms at the strain level and/or antibiotic-resistant strains (Croxatto et al. 2012; Sandrin et al. 2013; Hrabak et al. 2013).

Differentiation of bacteria beyond the species level with MS is challenging since the detected number of unique or characteristic biomarkers decreases as the similarity between microorganisms increases. As a result, the measurement requires a higher level of selectivity, mass spectral profile reproducibility (relative peak intensities), and mass accuracy. For MS-based measurements this may imply strategies involving additional sample preparation steps (e.g., protein extraction, digestion),

the inclusion of a separation step (fractionation, LC), extending the mass range of the analysis (detection of higher MW biomarkers), increasing the selectivity of the MS measurement (MS/MS), or including enzyme substrates or stable isotope reagents (Fig. 2.1).

Profile-Based Techniques for Strain-Level Differentiation

These measurements are attractive since they require the simplest form of instrumentation available, a MALDI-TOF-MS operated in the linear mode ($m/z \sim 2000$–20,000; Fig. 2.3a). Ideally, for profile-based differentiation, a high degree of reproducibility is desired, along with high signal-to-noise ratio (S/N), to consistently produce protein profiles with distinct and unique features (specific) to each strain. The approach used to prepare the sample in MS has enormous consequences on the quality (S/N) and reproducibility of the resulting mass spectral profiles (signal relative intensities), and so this is an obvious experimental step to optimize. This is both true for ESI and MALDI; however, MALDI is more appropriate for samples containing complex mixtures and more robust to small differences in solvent composition and procedural steps than ESI. For the differentiation of microorganisms at the strain level, sample preparation techniques can increase both the reproducibility of the signal(s) and the range of biomarkers detected. In fact, it is generally agreed that the incorporation of a protein extraction step increases the rate of identification of bacteria at the species level, and in some cases at the strain level, (Croxatto et al. 2012; Clark et al. 2013; Lartigue 2013) especially for Gram-positive cocci (Alatoom et al. 2011). This is most likely due to the removal of other cellular components and salts that can cause signal suppression, thus improving the overall S/N of the mass spectrum.

In general, the use of profile-based MS techniques to differentiate microorganisms at the strain level has met with limited success as the presence of unique strain-specific biomarker(s) can be inconsistent and/or microorganism-dependent. It is usually the case for MALDI-MS profiling that the number, nature, and quality of the reference mass spectra improves the reliability of the identification at the species level (Calderaro et al. 2013). For example, Shao and coworkers (Zhu et al. 2013) improved the identification of several strains of *Haemophilus influenza* and *H. haemolyticus* after curating their mass spectral database. The original reference mass spectral database failed to identify any of the *H. haemolyticus* strains at the species level, but was able to do so after the database was updated with reference mass spectra. In addition, cluster analysis of the obtained mass spectra profiles (and using the standard protein extraction protocol) yielded a dendogram clustering showing clear differentiation of *H. haemolyticus* from *H. influenza*. In addition, *H. influenza* was further differentiated by geographical origin, that is, the Chinese strains were differentiated from those of foreign origin.

However, in most cases, different strains of the same species are correctly identified only at the species level by MALDI-MS profiling (Kolecka et al. 2013;

Kierzkowska et al. 2013). For example, in the comparison of two commercially available microorganism identification systems based on the MALDI-MS instrument and using the standardize sample preparation protocol (direct transfer method, vide infra), 54 streptococcal strains were correctly identified only at the species level (Karpanoja et al. 2014). Similarly, in the MALDI-MS analysis of 24 clinical isolates of the fungus *Trichophyton rubrum*, (Pereira et al. 2014) all strains were identified at the species level only, even after efforts to optimize the sample preparation step using different matrices, formic acid extraction, and/or sonication. Finally, in an attempt to identify bacterial strains related to normal and sensitive skin disorders with MALDI-MS profiling, (Hillion et al. 2013) no correlation was found between phylum, genus or bacterial species and the sensitive skin phenotype, even though all bacteria were correctly identified at the species level. It is worth pointing out that these examples illustrate the importance of reproducibility in the generation of replicate mass spectral profiles of bacteria, as small differences in profiles can yield information about strain differentiation. However, this approach can be limited by the overall reproducibility or variance of the resulting mass spectra (both from samples and in the database), and thus this strategy can benefit from a reduction in the total variance of the analysis (vide infra).

Although limited, several strategies have successfully differentiated microorganisms at the strain level using profile-based MALDI-MS and they include: (1) optimization of the sample preparation step and (2) optimization of growth conditions. Other approaches incorporating bioinformatics (Demirev et al. 2001; Tamura et al. 2013) data analyses will not be discussed in this section.

Optimization of the Sample Preparation Step Prior to MALDI-MS This area of research has received a lot of attention from investigators using MALDI-MS due to the pronounced effects that sample preparation has on the resulting mass spectral profiles, the low cost of implementing these changes (mostly reagents and solvents), and the relatively ease of customization depending on the sample type and/or application. However, it is generally agreed that acidic conditions followed by addition of an organic solvent is sufficient to access most of the ribosomal proteins detected in profile-based MALDI-MS measurements. Other approaches have been proposed to either increase the S/N of the profile and/or extend the MW range of detected proteins and include on-probe sample treatment with ethanol, (Madonna et al. 2000) use of additives (crown ethers or thymol), (Liu et al. 2007; Holland et al. 2014) and the implementation of a heating step (Horneffer et al. 2004; Prieto 2006). Two standard sample preparation protocols are currently used in conjunction with most commercially available MALDI-MS bacteria identification systems, and can be broadly classified either as direct transfer or protein extraction. In the direct transfer protocol, a bacterial colony is smeared directly onto the MALDI plate and overlaid with matrix solution, with bacteria inactivation and protein extraction being performed on the MALDI plate as the matrix solution evaporates (< 1 min). Improved identification rates in the direct transfer method were achieved for Gram-positive rods by incorporating a 70 % formic acid pretreatment (on-probe) prior to the addition of the matrix (Werner et al. 2012; Schulthess et al. 2014). In the protein

extraction protocol or ethanol-formic acid procedure, (Freiwald and Sauer 2009) an isolated bacterial colony is first washed with deionized/distilled water, followed by a 75% ethanol/water wash. This step is intended to remove any media contamination and inactivate the bacteria (without spore formation). The resulting pellet is re-suspended in equal volumes of 70% formic acid and acetonitrile (sequentially added, up to 20 μL final volume) mixed and centrifuged. An aliquot (~1 μL) of the supernatant is deposited onto the MALDI plate, dried and overlaid with α-cyano-4-hydroxycinnamic acid (CHCA) matrix dissolved in a 50% acetonitrile/water and 2.5% TFA. For pathogenic bacteria with spore formation, (Lasch et al. 2008) the TFA protein extraction procedure is recommended and involves an aggressive inactivation step with 80% TFA followed by 50% acetonitrile/water.

The use of protein extraction, along a well-curated mass spectral database, can provide accurate identification of many strains at the species level, and in some cases, differentiation at the strain level. However, in most of these situations the differentiation is based on the pattern of several mass spectral peaks and not on a unique biomarker ion. For example, work conducted by Calderaro and coworkers (2014) on the differentiation of *Leptospira* species at the serovar level studied a panel of 20 *Leptospira* reference strains representative of six species. The analysis was performed to supplement their microorganism MALDI-mass spectral database and samples were prepared by the ethanol-formic acid method described above. Using standard chemometrics tools included in commercially available MALDI-MS microorganism identification systems, the authors identified 20 distinct mass spectral signals, their combined pattern being responsible for the differentiation of 12 serovars of *Leptospira interrogans*. For the *L. borgpetersenii* species, discrimination at the strain level of three serovars was based on the unique pattern of five signals in their mass spectra.

Another example highlighting the limited success of the sample preparation step for strain differentiation is found in the work conducted by Huber et al. (Zeller-Peronnet et al. 2013) where 24 strains belonging to the species *Leuconostoc mesenteroides* and *L. pseudomesenteroides* were analyzed by MALDI-MS profiling. Discrimination of the protein profiles by principal component analysis (PCA) generated three distinct clusters, but only half of the microorganisms studied were reliably discriminated at the strain level. The protein profiles in this study were generated from samples prepared by initially subjecting the bacteria suspension to lysozyme digestion (37 °C, 30 min), followed by a standard protein extraction protocol. It was determined that subjecting Gram-positive bacteria to enzyme digestion with lysozyme provides additional lysing of the thick peptidoglycan layer of the cell wall, (Giebel et al. 2008) thus making intracellular proteins more accessible to the MALDI matrix. The authors found that the lysozyme treatment improved only the reproducibility of the profiles (about a 10% improvement in the correlation coefficient), but they did not observe signals at higher *m*/*z*'s reported in other studies (Giebel et al. 2008; Vargha et al. 2006). This discrepancy could be attributed to various experimental factors, including the analysis of different bacteria by each group. However, these results highlight potential issues of irreproducibility in inter-

laboratory studies and limitations of approaches that introduce biological reagents (enzymes) which are prone to biological activity losses and are affected by storage and experimental conditions (e.g., pH, ionic strength, time).

Finally, two examples from the literature illustrate the dependency of strain-level differentiation by MALDI-MS profiles on the type of microorganism being analyzed. In a study using MALDI-MS profiling to differentiate seafood-borne pathogens, (Boehme et al. 2013) authors found mixed levels of success for species-level and strain-level identification. Even though the goal of this investigation was to compare MALDI-MS with 16S rRNA sequencing for their ability to identify food-borne pathogens, the authors achieved species-level identification for all 120 bacterial strains tested with MALDI-MS, and in the case of *Bacillus subtilis* subsp. Spizizenii, subspecies-level classification was possible. Equally, in the analysis of beer-spoiling *Lactobacillus brevis* strains, Behr et al. (Kern et al. 2014) compiled 17 strains of *L. brevis* varying in their environmental source (e.g., brewery vs. sour-dough) and their ability to grow (and spoil) different beers (Lager, Pilsner, etc.). Samples were grown in standardized media and prepared by the ethanol-formic acid protein extraction protocol prior to MALDI-MS analysis. A set of highly reproducible signals allowed the successful assignment of 90 % of the mass spectra collected to the correct strain. Misclassifications were attributed to either highly similar mass spectral patterns or mass spectral patterns with low number of peaks; however, this set of microorganisms strains was always classified correctly for their ability to either strongly or weakly spoil beer.

Optimization of Growth Conditions In this strategy, differentiation of microorganisms at the strain level by MALDI-MS profiles is achieved with the aid of judiciously chosen set of growth conditions. In one such study, the effect of growth conditions on the ability of MALDI-MS profiling to differentiate acetic acid bacteria (AAB) at the strain level was investigated (Wieme et al. 2014). Investigators found that growth medium effects on the mass spectral profile do not affect differentiation at the species level, but rather enhance the level of differentiation at the strain level. For example, eight strains of *Gluconobacter oxydans* were grown in acetic acid medium (AAM), yeast–peptone–mannitol (YPM) agar, and glucose–yeast (GY) agar and their MALDI-mass spectra compared for shared and strain-specific peaks. The results showed that only 7 % of the peaks were consistently present in all mass spectra, regardless of the growth medium used. In addition, it was observed that the number of strain-specific peaks varied from 3–4 with different growth medium, although none were observed when bacteria were grown in GY agar. This approach presents an effective, yet relatively simple and economic way to differentiate a specific set of bacteria at the strain level, for example, antibiotic-resistant strains or enterohemorrhagic serotypes of *Escherichia coli* (vide infra). However, its universal applicability is limited since the effects of growth media on the mass spectral pattern cannot be predicted and thus this approach would require considerable testing and development prior to its implementation for each target microorganism.

Increasing the Reproducibility of the MALDI-MS Measurement

The existence of so-called hot (or sweet) signal spots within a MALDI sample has long been recognized and is mostly due to the inhomogeneous distribution of the analyte within this matrix/sample preparation, (Horneffer et al. 2001) especially when the sample is prepared by a manual/pipette dried-droplet method (i.e., sample deposited and dried first followed by matrix) (Dai et al. 1996). Accordingly, this variance can be mitigated through an increase in the number of measurements, either by averaging a large number of laser shots and/or increasing the number of MALDI sample preparations being analyzed (Szájli et al. 2008). These strategies are already being incorporated in studies aimed at the analysis of microorganisms with MALDI-MS, as most protocols collect about 20–40 replicates for each bacterial sample being analyzed, and average 200–400 laser shots/spectrum. As a result, these measurements most likely have reached the limit of reproducibility that can be achieved with standard manual sample preparation methods.

The heterogeneous nature of the MALDI matrix as well as the uneven analyte distribution within it have been identified as one of the major sources of variance in signal strength between spots of a single MALDI sample preparation. The effect of this uneven distribution on the variance of MALDI mass spectral profiles of bacteria was quantified by using a spray-based method to homogeneously deposit *E. coli* samples (suspended in a CHCA matrix solution) onto the MALDI plate (Fig. 2.6) (Toh-Boyo et al. 2012).

This approach resulted in bacteria being evenly distributed across the deposited sample *(Caution: the spray method is not suitable for clinical samples!)*. Subsequent MALDI-MS analyses of these homogenous sample/matrix preparations yielded highly reproducible mass spectra, regardless of the spatial coordinates of the laser shot on the sample. When compared to the manual/pipette dried-droplet

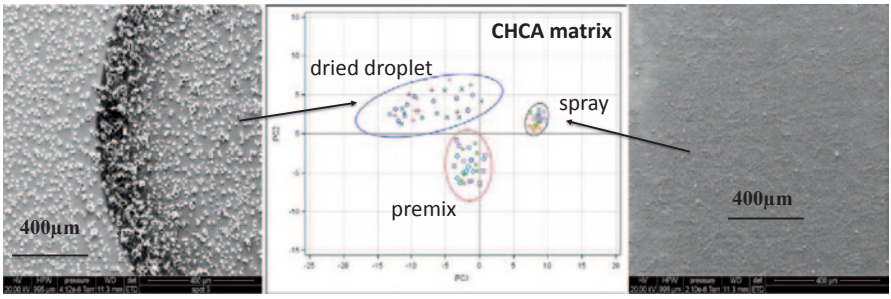

Fig. 2.6 PCA comparing the effect of matrix morphology on the reproducibility of bacteria MALDI-mass spectral profiles. All analyses were performed with *E. coli* (K-12) deposited by a spray-based method (uniform matrix deposition) and two manual pipette methods: dried droplet (simulating the direct transfer method) and premix (where a suspension of bacteria in matrix solution is deposited onto the MALDI plate). Ellipses represent the 95 % prediction space of the PCA clusters of replicate mass spectra for each deposition method (30 mass spectra/cluster). (See text for further details; Adapted from Toh-Boyo et al. 2012, copyright American Chemical Society)

method (equivalent to the direct transfer method), the spray method resulted in a 90% reduction of the total variance of the measurement. Most surprising was the resulting 60% reduction in the variance by the premix deposition method, where bacteria are suspended in the matrix solution prior to deposition, when compared to the manual dried-droplet method.

In principle, this increase in reproducibility would allow for the detection of small features and/or differences between the mass spectra of closely related microorganisms. Assuming the direct transfer method has a variance similar to that of the dried-droplet method used in this study (Fig. 2.6), it can be inferred that a significant reduction in the variance can be realized by simply premixing the intact bacteria with the matrix solution prior to its deposition onto the MALDI plate, rather than performing sequential depositions of bacteria followed by matrix. This improvement in the reproducibility would also be expected to be observed in the protein extraction protocols, adding only a single dilution step in the overall procedure (1:1 sample/matrix). However, to date, all standard protocols (Freiwald and Sauer 2009) used in profile-based MALDI-MS analyses implement a dried-droplet approach to deposit the sample onto the MALDI plate.

Increasing the Selectivity in Protein Biomarkers Detection

The profile-based MALDI-MS approach to identify and differentiate microorganisms, although powerful in the analysis at the species level, has met with mixed success at strain-level differentiation. This limitation stems in part from the fact that under the current experimental conditions ribosomal proteins are serendipitously detected, with the bulk observed in the mass range of 2000 to ~18,000 Da. For example, in *E. coli* there are 55 ribosomal proteins of known sequence that vary in MW from 4,400 to 61,200 Da (Wittmann 1982; Stelzl et al. 2001). Of these, about 80% (~44 proteins) have a MW within the detected mass range in the MALDI-MS measurement. However, this represents a small fraction of the available proteome in bacteria and thus severely limits the selectivity of the method. Case in point: it is known that *E. coli* has 4288 protein-coding genes or open-reading frames (ORF) (Blattner et al. 1997; Han and Lee 2006). Experimentally, however, two-dimensional (2D) gels and nongel methods have roughly identified about 1600 proteins in *E. coli,* (Corbin et al. 2003) with a MW range of 10^3–10^5 Da and *pI* values ranging from 4 to 12 (Han and Lee 2006). Therefore, it can be estimated that profile-based MALDI-MS measurements roughly detect only a small fraction of the possible protein pool in bacteria, or about 2.8% (44/1600) of the total detectable proteins in *E. coli*.

It is thus reasonable to argue that an increase in the selectivity of the method in order to detect a wider range of the microbial proteome will lead to the differentiation of closely related microorganism, as proteins responsible for unique genotypic traits would be detected. This point can be best illustrated with work by Murugaiyan and coworkers (2013), where they identified the protein expression levels between

pathogenic and nonpathogenic strains of the alga *Prototheca zopfii* (using 2D-gels and peptide mass fingerprinting, PMF, by MALDI-MS). Their results indicated that proteins responsible for differentiation of genotype 1 vs. 2 (nonpathogenic vs. pathogenic) were proteins related to energy, carbohydrate metabolism, and signal transduction (interestingly, ribosomal proteins remained unchanged between these two genotypes). One of the proteins upregulated in the pathogenic strain was a cyclic nucleotide-binding domain protein with an average MW of 51 kDa, a protein known to be associated in bacterial adaptation to a changing environment and well above the mass range usually analyzed in MALDI-MS profiling of microorganisms. Because the limited dynamic range in the proteins detected by MALDI-MS is due primarily to the ionization step (i.e., MALDI), merely extending the mass range of the TOF-MS will not overcome this limitation. Furthermore, approaches incorporating modified sample preparation protocols using additives and combinations of organic solvents have had limited and irreproducible success (vide supra).

In order to extend the MW dynamic range of detected proteins by MS (either ESI or MALDI), a separation step is often incorporated prior to detection. In the case of top-down (LeDuc et al. 2004; Zhou and Ning 2012) or bottom-up Zhang et al. (2013a) proteomics approaches, the incorporation of separation step is implemented in order to handle the highly complex protein and/or peptide mixture. In bottom-up proteomics-based measurement, the protein sample is digested into smaller peptides and the protein identity (and its biological source) inferred from the analysis of these peptides. In top-down proteomics-based measurements, the intact protein identity is derived directly from its analysis. In both cases, instrumentation capable of MS/MS measurements is required, although not all MS/MS instruments can perform both top-down and bottom-up analyses, and depending on the ionization mode and ion dissociation mode, the upper mass range limit is different (vide supra). Common to these proteomics measurements is the implementation of a separation step, mainly LC, prior to MS and MS/MS measurements.

Other top-down (Demirev et al. 2005; Fagerquist et al. 2010; Fagerquist et al. 2009; Fagerquist 2013) and bottom-up (Yao et al. 2002a, b) proteomic approaches *without* a separation step utilize MALDI in conjunction with a tandem MS or MS/MS—e.g., TOF/TOF-MS system in Fig. 2.3b—to increase the specificity of the analysis. These approaches do not extend the number of protein biomarkers detected, but rather they can analyze individual protein biomarker signals, and in principle a mixture of bacteria, provided representative signals from all species are generated during the MALDI process. This approach implements the same rapid protein extraction protocol as in the profile-based techniques.

Top-Down Proteomic Approaches

Strategies implementing LC-MS and LC-MS/MS can extend the number of proteins accessible in the microbial proteome for the purpose of strain-level differentiation. In top-down proteomics, the microbial sample is usually processed in

order to extract and isolate a highly enriched protein fraction. Top-down proteomic approaches can implement both MALDI and ESI, as both of these ionization modes can be interfaced with MS/MS instrumentation. Furthermore, both MALDI and ESI can be coupled with LC separation for additional selectivity in the analysis, albeit for MALDI the LC separation step is performed in an offline mode (Marcus et al. 2007; Bodnar et al. 2003; Basile et al. 2005; Maltman et al. 2011). The following discussion will focus on coupling LC with ESI-MS for the analysis of closely related microorganisms.

As stated earlier, ESI (and MALDI) is limited in its ability to ionize every component present in a complex mixture, as in crude bacterial protein extracts, and thus approaches aiming at increasing the dynamic range of proteins detected must incorporate a separation step (e.g., 2D gel, LC). However, strategies incorporating gel-based separation steps, although possessing large peak capacities, are time-consuming and are not amenable for high-throughput analyses of microbial samples. On the other hand, LC-based methods are suitable for high sample throughput, while providing the required selectivity to detect a wide range of proteins present in the sample. Because the analysis is usually carried out in a broad spectrum mode, that is, targeting all possible proteins, an aggressive lysis step is usually implemented. In addition, since both MALDI and ESI are sensitive to high concentrations of ionic species in solution (ESI in particular), physical methods are usually preferred (e.g., pressure, sonication, beads) that preclude the addition of high concentrations of lysis agents. In some instances the released DNA in solution is eliminated by the addition of DNase I. The final protein fraction must be devoid of any solid or suspended matter as it may obstruct/clog valves, syringes and frits present in the LC system. All these steps add to the overall sample preparation time, often requiring several hours of manual labor. Several compilations dealing with sample preparation methods for the analysis of proteins with LC-MS techniques can be found elsewhere, (Aguilar 2004; Shah and Gharbia 2010) and they will not be discussed in detail in this section.

Unlike MALDI-MS, the analysis of intact proteins by LC-(ESI)-MS requires additional processing steps of the mass spectral data in order to extract protein MW information. This is due to the multiple-charged nature of the mass spectral signals of proteins under ESI conditions. This process is best illustrated in the analysis of *Salmonella* spp. protein extraction by LC-ESI-MS. Figure 2.7 shows the initial LC-MS chromatogram, which is deconvoluted, in 30 s time windows, throughout the entire chromatographic period (e.g., 80 min in this example).

That is, the related m/z values from an eluting protonated protein (i.e., $[M+nH]^{n+}$) are converted to a single mass value for the neutral intact protein (i.e., M) (McFarland et al. 2014; Williams et al. 2002). The resulting mass profiles for each 30 s window are combined into a single profile showing peak intensity and mass of the protein (retention time information is also preserved during this data analysis, but not plotted). This intact protein expression profile is easy to interpret, since it represents all of the proteins detected in the sample.

Several advantages result from this approach. First, because LC is incorporated into the ESI-MS analysis, suppression effects are minimized leading to the

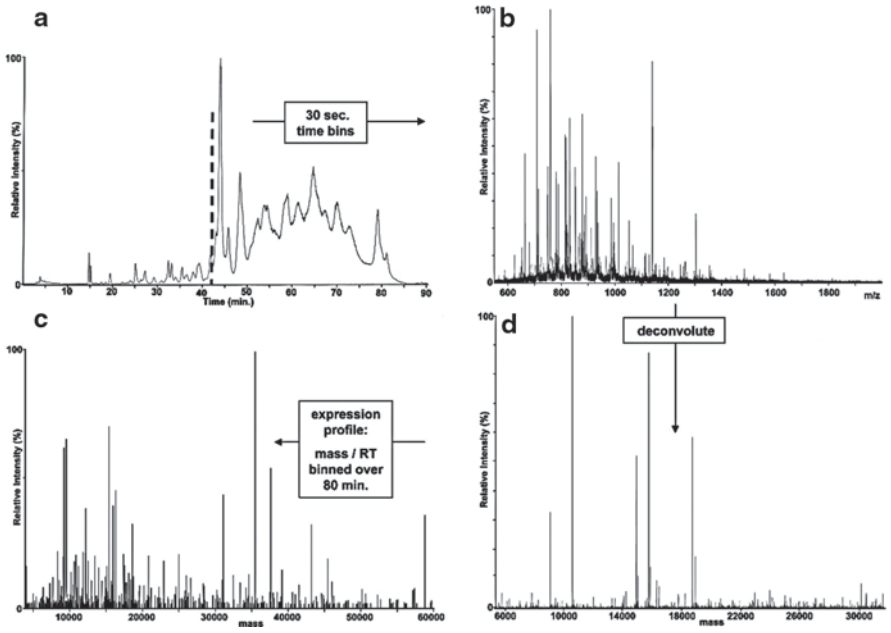

Fig. 2.7 Generation of protein mass profiles for *Salmonella* spp. using LC-ESI-MS. Multiple-charged signals from proteins within a 30 s time window are deconvoluted into single mass values (mass of neutral protein). The process is repeated for the entire chromatographic period (~80 min) and combined into a single mass profile. (Adapted from McFarland et al. 2014, copyright American Chemical Society)

detection of a larger number of proteins that otherwise would not be detected with MALDI-MS alone. Figure 2.8 illustrates this advantage where a comparison is made between the MALDI-mass profile and the LC-(ESI)-MS protein expression profile of *Shigella sonnei,* the latter showing enhanced detection of several proteins above 15 kDa (Everley et al. 2008). This approach was later used to successfully differentiate several strains and isolates of pathogenic and nonpathogenic *E. coli* (Mott et al. 2010).

A second advantage of the LC-ESI-MS approach is the increased mass accuracy of the measurement, making possible the detection of small mass differences between proteins. These mass differences can be equivalent to single-nucleotide polymorphisms (SNP) mutations or post-translational modifications. Recent work by McFarland and coworkers (2014) best illustrates the implementation of LC-(ESI)-MS (intact protein mass) and MS/MS top-down analyses to bacteria differentiation at the strain level. Proteins extracted from bacterial samples of *Salmonella typhimurium* (strain LT2) and *S. heidelberg* (strain A39) were first separated by reversed-phase (RP) LC and the eluent analyzed directly by ESI-MS using Q-TOF MS system (operated in the full-scan mode or MS). Following the data processing

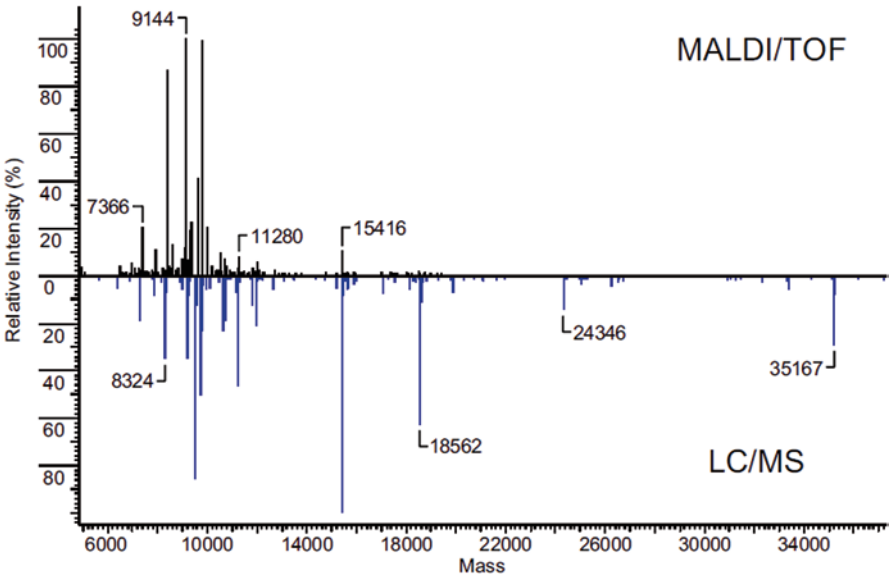

Fig. 2.8 Comparison of MALDI-MS profile (protein extraction; sinapinic acid matrix) with the LC-MS mass profile for *Shigella sonnei*. The *x*-axis represents the mass of the neutral proteins, M, and not the *m*/*z* of the $[M+nH]^{n+}$ ions in the ESI- or MALDI-mass spectra. (Adapted from Everley et al. 2008, with permission from Springer)

shown in Fig. 2.7, the resulting deconvoluted mass spectra were then displayed as intact protein expression profiles, in a mirrored configuration for easy comparison (Fig. 2.9). Although the similarity of these two serovars in terms of their protein expression profiles (mass maps) make them almost indistinguishable, close inspection reveals that several proteins showed detectable mass shifts (highlighted in Fig. 2.9) between the samples. These mass shifts most likely represent protein products of SNP containing genes that differentiate these two strains. The identification of proteins in Fig. 2.9 was accomplished in a second analysis by LC-MS/MS of the intact proteins with an orbitrap mass analyzer (Fig. 2.5).

A third advantage of this approach, when combined with top-down and bottom-up proteomic measurements, is the ability of "reverse engineering" unique segments within a protein sequence into polymerase chain reaction (PCR) primers that have specificity toward a desired phenotypic trait (Williams et al. 2005). This is especially useful for organisms with unsequenced genomes. Overall, the extended dynamic range of detected and identified intact proteins with LC-MS and MS/MS allows for an increase in the analysis selectivity among different microbial strains. However, development of these techniques for clinical analyses would have to include automated sample preparation and analysis in order to achieve a high sample throughput. In general, its implementation is expected to provide a flexible platform for enhanced discrimination of closely related microorganisms, including antibiotic-resistant strains, which will be presented next.

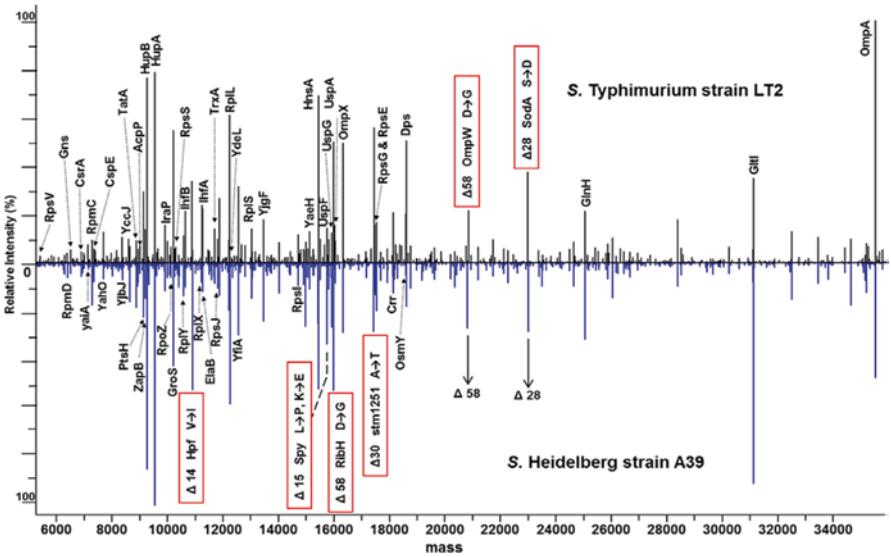

Fig. 2.9 Mass profiles obtained by LC-MS analysis of protein extracts of *S. typhimurium* strain LT2 and *S. heidelbergh* strain A39. The *x*-axis represents the mass of the neutral proteins, M, and not the *m*/*z* of the $[M+nH]^{n+}$ ions in the ESI-mass spectra. (Adapted from McFarland et al. 2014, copyright American Chemical Society)

Differentiation and Detection of Antibiotic-Resistant Bacteria

Clinically relevant is the detection of antibiotic-resistant strains, as accurate information about the infectious microorganism can result in improved patient outcomes and a concomitant reduction in health care costs. Knowledge of the resistance mechanism is key in order to develop accurate and specific MS-based methods for the detection of antibiotic (or antimicrobial)-resistant microorganisms. Bacteria can acquire antibiotic resistance via these five mechanisms: (i) mutation of the target site, (ii) enzymatic modification of the antibiotic, (iii) active efflux of antibiotics from the cell, (iv) restricted permeability of antibiotics to the active site, and (v) acquisition of an alternative metabolic pathway(s) insensitive to the antibiotic (Mc Dermott et al. 2003; Vranakis et al. 2014). Accordingly, several MS-based techniques have been developed to detect antibiotic resistance in bacteria derived by some or all of these mechanisms. These detection strategies include: (1) profile-based MALDI-MS of intact cells or protein extracts, (2) detection of the enzymatic modification of the antibiotic (a.k.a., enzyme activity methods), (3) direct detection of the enzyme degrading the antibiotic, and (4) profile-based MALDI-MS of intact cells grown in stable isotope/selective media. A brief discussion of each technique will follow with representative examples from the published literature.

Profile-Based MALDI-MS of Intact Cells or Protein Extracts In this approach commercial MALDI-MS systems are used to differentiate antibiotic-resistant strains

from their susceptible counterparts. The profiles generated from these two samples are usually indistinguishable in terms of unique biomarkers present (in the mass range of 2000 to 20,000 Da). That is, no unique biomarker(s) is responsible for antibiotic resistance differentiation within this mass range analyzed. This is understandable since most of the enzymes responsible for antibiotic resistance (carbapenemase, (Queenan and Bush 2007) vide infra) have MWs in the 28–31 kDa range. However, several studies have been successful at this differentiation by a careful control of the experimental conditions (Jackson et al. 2005; Goldstein et al. 2013) either using the direct transfer method (Goldstein et al. 2013; Majcherczyk et al. (2006) or the standard ethanol-wash and formic acid/acetonitrile protein extraction protocol, (Wybo et al. 2011; Griffin et al. (2012) while performing the MS analysis in the 2000–20,000 Da mass range. The method relies on the detection of subtle differences between mass spectral profiles of susceptible and resistant strains of the microorganisms, which must be present in the mass spectral database. For example, in the MALDI-MS differentiation of *Bacteroides fragilis* susceptible and resistant strains to the antibiotic meropenem, no unique signal was associated with resistance, but rather the two groups were discriminated based on the profiles of approximately 10 signals (Wybo et al. 2011). As a result, the profile-based MALDI-MS approach has shown very limited success as a universal method for antibiotic resistance detection and must be validated on a case-by-case basis. However, because this methodology is already in place in most clinical laboratories, it is expected that implementation of protein extraction protocols in conjunction with other procedures that reduce the variance of the measurement (vide supra) could lead to more confident differentiations between these closely related strains.

Enzyme Activity Via Antibiotic Degradation Product Detection This approach is based on monitoring the products of an enzyme-catalyzed reaction by MS (Gerber et al. 1999; Bothner et al. 2000; Gerber et al. 2001; Basile et al. 2002; Chennamaneni et al. 2014). The advantage of using MS over commonly used optical methods (e.g., UV-vis absorption or fluorescence) is that specific substrates labeled with chromophore molecules are not required, that is, substrates can be used in their native state, and products are differentiated from the substrate molecule simply by a characteristic mass change. In addition, given the resolution of most MS systems and a judicious choice of substrate molecules (MW's), multiple substrate–enzyme reactions can be monitored simultaneously with a single MS-based enzyme activity approach (Basile et al. 2002).

The application of MS-based enzyme activity to detect antibiotic-resistant bacteria was first reported by Hrabák et al. where carbapenemase activity was detected using MALDI-MS in viable intact cells of Enterobacteriaceae and *Pseudomonas* spp. (Hrabak et al. 2011) and later expanded to *Acinetobacter baumannii* (Hrabák et al. 2012). Carbapenemase activity detection in bacteria with MALDI-MS requires the incubation of viable bacteria (in a suitable buffer system, e.g., 20 mM Tris–HCl, 0.01 % sodium dodecyl sulfate (SDS), pH 7.0) with the substrate molecule, in this case, the antibiotic molecule (e.g., meropenem). This suspension is incubated for ~2 h at 35 °C, in which period meropenem molecules are enzymati-

cally hydrolyzed at the β-lactam moiety followed by decarboxylation. The bacteria suspension is then centrifuged and the supernatant is analyzed by MALDI-MS. The MALDI-MS analysis is performed in the mass range of 160–600 Da in order to detect the low MW products. The matrix 2,5-dihydroxybenzoic acid (DHB) was used as it has a very low chemical background in this low mass range. For example, for meropenem, the intact molecule (unmodified) is detected at *m*/*z* 384.16 (calculated monoisotopic mass of the $[M+H]^+$ ion), while the product (hydrolyzed and decarboxylated) is detected at *m*/*z* 358.18 (calculated monoisotopic mass of the $[M+H]^+$ ion; the sodium adduct ion, $[M+Na]^+$, is also detected at *m*/*z* 380.16). Mass spectra of susceptible strains of bacteria will show signals corresponding to the unreacted, intact antibiotic molecule, while resistant strains will show both the intact antibiotic and hydrolysis/decarboxylation product. Because the antibiotic molecules undergo spontaneous, but slow, hydrolysis (a.k.a., auto-hydrolysis), a background signal or measurement must be made in order to provide quantitative measure of the enzyme activity. This approach was successful in detecting antibiotic resistance from different carbapenemases (NDM-1, KPC-2, KPC-3, VIM-1, OXA-48, and OXA-162) in several microbial species (Hrabák et al. 2012).

Other laboratories have successfully implemented this technique to detect carbapenemase activity in several species of *Pseudomonas* and Enterobacteriaceae using the antibiotic (i.e., substrate) ertapenem (Burckhardt and Zimmermann 2011) and imipenem (Kempf et al. 2012; Alvarez-Buylla et al. 2013). A detailed characterization of this enzyme assay was conducted with *E. coli* cell lysate/extract (expressing chromosomally encoded AmpC β-lactamase) and using both a MALDI-TOF-MS and a MALDI-QQQ-MS in the MRM mode (see Fig. 2.4) to detect β-lactamase enzyme activity with the substrate penicillin G (PenG) (Hooff et al. 2012). The implementation of an SRM detection mode that allowed for accurate kinetic degradation studies was able to detect enzyme activity within 5–15 min of incubation time as well as establishing statistical parameters in terms of inter- and intraday reproducibility. A quantitative measurement of carbapenemase activity was also performed by incorporating a ^{18}O-labeled internal standard antibiotic molecule in conjunction with ESI-QQQ detection (where Q3 was a linear quadrupole ion trap, rather than a quadrupole mass filter), (Wang et al. 2013) increasing the accuracy, specificity and reproducibility of the measurement while at the same time reducing false positives due to auto-hydrolysis of the drug (substrate).

These analyses showed successful detection of carbapenem antibiotic resistance utilizing available instrumentation and without any special reagents (other than the antibiotic themselves). However, this analysis does require a separate sample preparation step, and instrumentation parameters, and thus may add to the overall sample preparation time and hinder high-throughput measurements. The ability to perform SRM measurements with these approaches can in principle provide a high-throughput platform for the detection of bacteria resistance toward multiple β-lactam drugs in a single measurement.

Direct Detection of Enzymes Responsible for Antibiotic Degradation/Resistance In this strategy antibiotic resistance in bacteria is established by the direct detection

of the carbapenem-hydrolyzing β-lactamase(s). Two studies will be described that demonstrate this strategy via a direct MALDI-MS measurement and a bottom-up proteomic approach.

Combined, MALDI with TOF-MS systems have a practical upper mass limit detection of ~60–160 kDa, depending on sample complexity and preparation, and so it is feasible to use MALDI-MS systems for the detection of proteins other than the ribosomal proteins used in profile-based identifications. Most carbapenemases have MW's that range between 28 and 31 kDa, and thus their detection by commercially available MALDI-MS is feasible. However, as mentioned earlier in this chapter, the prevalence of ribosomal proteins in the MALDI-mass spectrum profile of bacteria is a direct consequence of their abundance and ease of ionization. That is, detection of other proteins present in the sample may be suppressed by the presence of these ribosomal proteins. As a result, MALDI matrix and sample reparation conditions need to be optimized in order to detect higher MW proteins in these complex samples. Indeed, proof-of-principle work by Camara and Hays (2007) demonstrated that by optimizing the sample preparation and MALDI matrix it was possible to detect a 29 kDa β-lactamase in *E. coli*. The method used a sample preparation protocol that included a 0.1% TFA cell wash, followed by a protein extraction in formic acid/isopropyl alcohol/water (17:33:50 by vol.) and used sinapinic acid as the MALDI matrix (Wang et al. 1998). Although limited in scope and bacterial species analyzed, this study demonstrated the detection of higher MW proteins using a modified protein extraction protocol and without the use of LC prior to the MS analysis step. However, this approach may require optimization of the MALDI sample preparation step for each type of sample (e.g., Gram-type, genus, etc.), thus limiting its applicability as a universal detection protocol for known and unknown microbial samples.

The detection of the β-lactamase enzyme in bacteria was also accomplished via a bottom-up proteomic approach (Fig. 2.1) in order to provide increase specificity to the assay via protein identification. Two examples from the literature will be used to illustrate this approach. In the first report, Hu et al. detected β-lactam resistance in *Acinetobacter baumannii* (Chang et al. 2013) by implementing a bottom-up proteomic approach using a microwave heating-assisted trypsin digestion of the protein extract followed by RP LC-MS/MS. The authors were able to identify a unique tryptic peptide in all the β-lactam-resistant clinical isolates of *A. baumannii* tested. In addition, because the analysis was performed in a data-dependent mode, a protein distribution profile was also obtained that can be used to further classify the sample. In a second study involving bottom-up proteomics, Hensbergen and coworkers (Fleurbaaij et al. 2014) employed capillary electrophoresis (CE)-MS/MS (using a Qq-TOF-MS system) to detect antibiotic-resistant Gram-negative bacteria. A total 14 tryptic peptides unique to antibiotic-resistant bacteria were identified in this study, all derived from the OXA-48 and KPC carbapenemases.

Because these bottom-up proteomic approaches are conducted in a data-dependent mode, it is foreseeable that other β-lactamases can be identified. In addition, an increase in the detection and identification confidence level is accomplished by the detection of multiple peptides per protein. The discovery of unique peptide bio-

markers corresponding to β-lactamases could be used to develop targeted analyses using an MRM mode in a QQQ instrument for increase in specificity and rapid data analysis. However, given the stochastic nature of proteomic-based approaches, (Zhang et al. 2013b) in particular those involving a separation step and MS/MS detection, avoiding or decreasing the number of false-negative outcomes may require the use of internal standards, detection of multiple biomarkers within a measurement, and tests using selective growth media. This shortcoming will also require the analysis of several biological replicates, which unfortunately increases the analysis time mostly due to the added individual chromatographic steps.

Profile-Based MALDI-MS with Stable-Isotope/Selective Growth Media In this approach MALDI-MS profiles are obtained for bacteria grown in selective growth media containing antibiotic and with nutrients enriched with heavy (stable) isotopes of ^{13}C, or both ^{13}C and ^{15}N. Operationally, antibiotic-resistant bacteria would be able to grow in the presence of antibiotic, and during this process incorporate nutrients with the heavy isotopes, which are used in the biosynthesis of heavy homologs of protein biomarkers. By comparing these results with those from the same analysis performed in control media (i.e., natural isotope abundance) and without the antibiotic, mass shifts between these protein signals can be used to establish antibiotic resistance. Two approaches have been demonstrated using different growth media and data analysis/algorithm. In the first published study, bacteria were grown in 98% ^{13}C isotope-enriched media (and control media with natural isotope abundance) (Demirev et al. 2013). In a second approach, (Sparbier et al. 2013; Jung et al. 2014) samples were grown in media containing "heavy" lysine (^{13}C and ^{15}N enriched lysine) and control or "light" media (containing naturally occurring lysine). In both of these schemes, it is essential to measure a control mass spectrum to establish mass shifts of the proteins biosynthesized in the presence of heavy isotopes. The main advantage of these analyses is that they provide a universal method to detect *any form of antibiotic resistance* in bacteria.

Conclusions

The use of MS-based techniques for the differentiation of closely related microorganisms requires the close interplay of biochemical knowledge of the sample and the capabilities and requirements of MS hardware. The main information derived from MS analysis, m/z of the ions produced during the ionization process, provides a unique and specific set of biomarkers for the differentiation of microorganisms. Because of the wide acceptance of MALDI-TOF-MS systems by the microbiology community, it is reasonable to channel efforts at developing new analytical methods for the detection of closely related microorganism based on this platform, as is the case for enzyme activity (Hrabak et al. 2011) and stable-isotope media (Demirev et al. 2013) tests outlined above. In addition, the development of methods involving new sample preparation protocols and using MALDI-TOF-MS platforms is worth

pursuing, given the occurrence of this instrument in many laboratories. For example, efforts should continue to explore methodology intended to inactivate and/or extract proteins (Machen et al. 2013) that can enhance the ability to differentiate microorganisms at the strain level. Finally, approaches measuring metabolite (and their levels) should also be revisited and/or developed as new evidence points to the effect of antimicrobial drug resistance on the metabolomic phenotype of bacteria, yielding to the discovery of some unique metabolites (Derewacz et al. 2013). Their analysis can be performed with a wide range of accessible MS instrumentation, including MALDI-TOF-MS and bench-top ESI-MS systems.

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