Chapter 6
Maximizing the Taxonomic Resolution of MALDI-TOF-MS-Based Approaches to Bacterial Characterization: From Culture Conditions Through Data Analysis

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Introduction

Bioterrorism, infectious disease, and microbial contamination of food and water threaten public health and safety (Bain et al. 2014; Chiu 2014; Ferreira et al. 2014; Murray 2010; Trafny et al. 2014). Infectious bacterial diseases cause nearly 20 million deaths annually (Chiu 2014; Lazcka et al. 2007). Therefore, the development of rapid, reliable, and sensitive methods for microbial identification is critically important in environmental monitoring, clinical microbiology, as well as water quality and food safety.

Bacterial resistance to antibiotics has increased significantly recently (Gentile et al. 2014; Martin-Loeches et al. 2014). Antibiotic-resistant strains, such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), and multidrug resistant bacteria, for example, bacteria with New Delhi Metallo-beta-lactamase-1 (*NDM*-1) gene (Eells et al. 2013; Epstein et al. 2014; Holland et al. 2014b; Morgan et al. 2014; Epstein et al. 2014) complicate the treatment of infections (Livermore 2012). Rapid determination and detection of antibiotic resistant strains play an important role in therapy (Niederman 2009) and is necessary for preventing transmission of such pathogens (Grundmann et al. 2010). This highlights the needs for rapid approaches to differentiate antibiotic-resistant from antibiotic-sensitive strains of pathogenic microorganisms. Strain-level information is also critically important when identification of strains with increased virulence or expanded host range is sought (Li et al. 2009). As a result, rapid bacterial strain typing, or identifying bacteria at the strain level, has become increasingly important in modern microbiology.

Bacterial taxonomic levels remain highly debated in the literature (Gao and Gupta 2012; Staley 2006). The term "strain" is used in this chapter to refer to a taxo-

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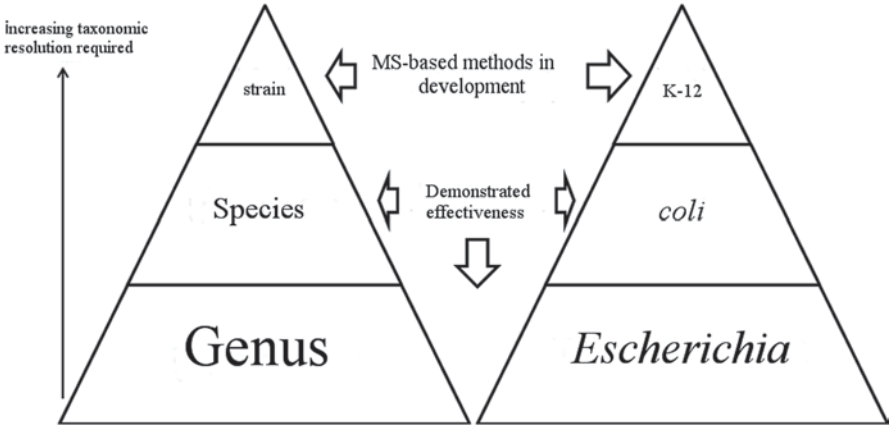

Fig. 6.1 Increasing taxonomic resolution is required to reliably characterize bacteria when moving from the genus level (e.g., *Escherichia*) to the subspecies (e.g., K-12) level. Well-described and effective methods exist for many applications at the genus and species levels, while strain-level applications require additional methodological development and optimization

nomic level more specific and exclusive than species, which includes all subspecies taxa (Fig. 6.1).

Traditionally, methods to type bacteria are classified into two groups: phenotyping and genotyping. Bacterial phenotypes can be determined by assessing the morphology of bacterial colonies on solid media surfaces, gram staining, biochemical/metabolic patterns, immunology-based methods, and antibiotic susceptibility. These methods often do not provide enough information to differentiate closely related strains. Discrimination of strains based on comparison of genetic variation is widely used. Bacteria can be classified using DNA fingerprinting, DNA sequence information, and microarrays (Li et al. 2009). DNA fingerprint-based methods analyze patterns of DNA bands (fragments) which are generated by digestion of genomic DNA using restriction enzymes, amplification of DNA, or by a combination of both. Such methods include pulsed-field gel electrophoresis (PFGE; Spanu et al. 2014), restriction fragment length polymorphism (RFLP; Perez-Boto et al. 2014), repetitive sequencing-based polymerase chain reaction (rep-PCR; Nucera et al. 2013), multiple-locus variable number tandem repeat analysis (MLVA; Shan et al. 2014), and denaturing/temperature gradient gel electrophoresis (DGGE/TGGE; Xiao et al. 2014). Each of these genotyping methods can provide quantitative, accurate information about the unknown bacteria; however, they are time consuming, laborious, and technically demanding. Some methods, such as microarray-based methods, are also particularly expensive (Li et al. 2009).

For more than two decades, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been shown to be a rapid and effective tool to profile bacteria at the genus and species levels (Dallagassa et al. 2014; Demirev and Fenselau 2008; Freiwald and Sauer 2009; Giebel et al. 2010; Welker and Moore 2011). The utility of this approach to profile bacteria at the strain level has not been as clearly demonstrated, in part, because similar bacteria tend

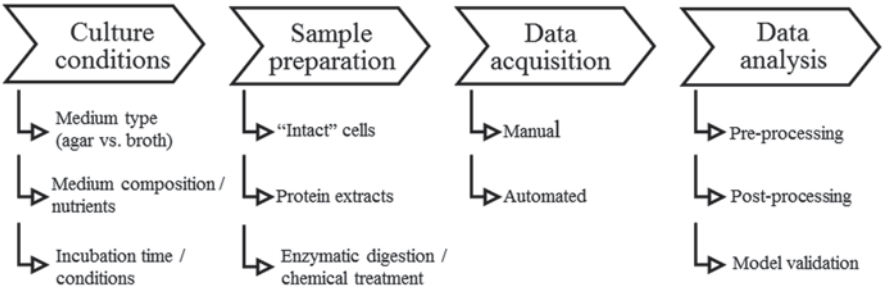

Fig. 6.2 A generic workflow from cultivation through data analysis to characterize bacteria using MALDI-TOF MS. At each step of the workflow, different approaches have been employed and can be optimized to maximize taxonomic resolution

to produce remarkably similar MALDI-TOF profiles that often do not allow facile differentiation of individual strains from one another. The focus of this chapter is on recent developments and the state of the science of maximizing the taxonomic resolution of MALDI-TOF MS-enabled characterization of bacteria. In particular, the chapter focuses on recent approaches employed throughout the MALDI-TOF MS workflow—from culture conditions, sample preparation, data acquisition, through data analysis—that affect and can be optimized to enhance the performance of MALDI-TOF MS-based characterization of bacteria at the strain level (Fig. 6.2).

Overview of MALDI-TOF MS Profiling of Bacteria

Mass spectrometry was first used for microbial characterization by Anhalt and Fenselau in 1975 (Anhalt and Fenselau 1975). By directly inserting lyophilized cells into a double-focusing mass spectrometer (CEC 21-110), *Staphylococcus epidermidis* and *Staphylococcus aureus* produced distinguishable mass profiles. MALDI-MS was first introduced for analysis of high mass peptides and proteins in the late 1980s (Karas et al. 1987). A few years later, protein profiles of lysed and intact bacterial cells, for example *Escherichia coli,* were analyzed by MALDI-TOF MS (Cain et al. 1994; Holland et al. 1996). Results showed that bacteria could be easily distinguished based on these "fingerprint" mass spectra. Since that seminal work, a staggering number and diversity of medically and environmentally relevant bacteria have been profiled using MALDI-TOF MS (e.g., Dallagassa et al. 2014; Ge et al. 2014; Giebel et al. 2008).

Library-Based Approaches

The most commonly employed approach to characterizing bacteria using MALDI-TOF MS involves comparing mass spectra of unknown bacteria to spectra in databases that contain spectra of known reference bacteria (Fig. 6.3a). This library-

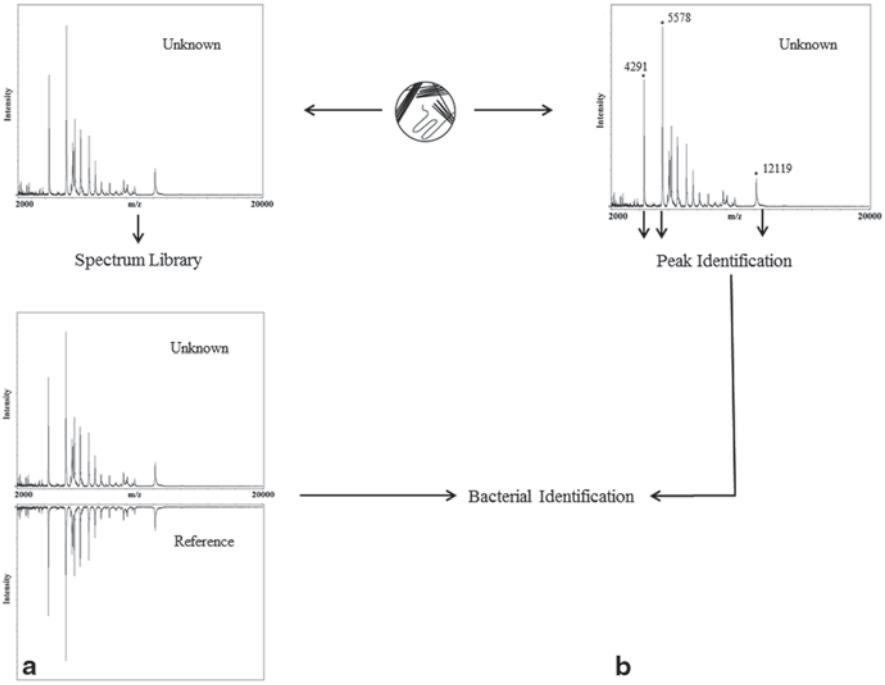

Fig. 6.3 MALDI-TOF MS-enabled characterization of unknown bacterium using **a** library-based approaches and **b** bioinformatics-based approaches

based strategy is popular because of its ease of use and the high speed of data collection (Chiu 2014; Fenselau 2013; Sandrin et al. 2013) . The reproducibility of mass spectra of unknown bacteria must be assessed before matched to mass spectra in the databases, and high reproducibility is critically important, particularly in strain-level applications.

Typically, bacteria are streaked onto agar plates (Grosse-Herrenthey et al. 2008; Pennanec et al. 2010; Stets et al. 2013; Wang et al. 2014) or grown in liquid broth (Han et al. 2014; Wensing et al. 2010; Xiao et al. 2014b). After harvesting from broth or agar media, bacterial cells are inactivated and prepared for MALDI analysis (Freiwald and Sauer 2009). Both intact cells (Han et al. 2014; Niyompanich et al. 2014; Wang et al. 2014) and cell protein extracts (Goncalves et al. 2014; Kopcakova et al. 2014; Stets et al. 2013) have been widely used. In the intact cell approach, cells are often directly mixed with MALDI matrix, such as sinapinic acid or alpha-cyano-4-hydroxycinnamic acid, and the cell-matrix mixtures are deposited onto the target for analysis (AlMasoud et al. 2014; Helmel et al. 2014). Cultures/cell suspensions have also been applied directly to the MALDI target plate and overlaid with matrix (Carbonnelle et al. 2007; Christner et al. 2014; Han et al. 2014). In protein extract-based approaches, cells are lysed using either physical (Fujinami et al. 2011; Sun et al. 2006; Teramoto et al. 2007a) or chemical (AlMasoud et al. 2014;

Wieme et al. 2014b; Zhang et al. 2014b) methods to release the contents of the cells into the supernatant. The supernatant containing the extracted proteins is then either overlaid (Wenning et al. 2014; Wieme et al. 2014b) or mixed (AlMasoud et al. 2014) with MALDI matrix and deposited onto the target. Excellent and comprehensive reviews of various sample preparation methods (Šedo et al. 2011a) as well as a detailed protocol for sample preparation for profiling bacteria using MALDI-TOF MS (Freiwald and Sauer 2009) are available.

Reference spectra are generally collected using the linear detector of the MALDI TOF MS. Both automatic data acquisition using software (e.g., AutoXecute in Bruker's FlexControl software) to control the mass spectrometer (Eddabra et al. 2012; Schumaker et al. 2012; Zhang et al. 2014a) and manual data acquisition (Khot et al. 2012; Schumaker et al. 2012) have been used to collect spectra. Automated data acquisition can enhance the high-throughput nature of MALDI profiling, while manual data acquisition has been shown to yield mass spectra with higher quality and reproducibility. Typically, spectra are collected over a mass range from 2 to 20 kDa (Salaun et al. 2010; Stets et al. 2013; Thevenon et al. 2012), while broader mass ranges, for example, 1 Da–100 kDa (Jackson et al. 2005) and 1 Da–20 kDa (Hettick et al. 2006), and narrower mass ranges such as 500 Da–10 kDa (Keys et al. 2004; Rajakaruna et al. 2009) and 7–10 kDa (Sauer and Kliem 2010) have also been employed. In automatic data acquisition, users must specify threshold values of several instrument operation parameters, including minimum base peak intensity, resolution, signal-to-noise ratio, and the number of peaks accumulated. These operation parameters can be optimized to increase the quality and reproducibility of the resulting mass spectra (Zhang et al. 2014a). After collecting mass spectra from a large collection of bacterial strains, the spectra are processed and analyzed to obtain information (e.g., similarity coefficients and potential biomarker peaks) to characterize unknown bacteria. A reference spectrum of a species is generated by summarizing the processed spectra of technical and/or biological replicates of the species. The mass spectra of unknown bacteria are then compared with the reference spectra for characterization using a variety of metrics, including manufacturer-defined algorithms (e.g., BioTyper scores), Pearson product–moment correlation coefficients, and jackknife values, which are described more fully later in this chapter.

Bioinformatics-Enabled Approaches

In contrast to library-based approaches which typically do not involve identification of the biological nature/origin of particular peaks, bioinformatics-enabled approaches identify peaks in MALDI profiles to characterize unknown bacteria (Fig. 6.3b). Bioinformatics-enabled approaches are commonly applied to bacteria with sequenced genomes. Two methods have been used in bioinformatics-enabled approaches: bottom-up and top-down methods. Bottom-up methods involve digestion of proteins using enzymes, such as trypsin, prior to MS analysis. The enzymes cleave at well-defined sites (e.g., after every arginine and lysine, in the case of tryptic digestion) of the proteins to create complex peptide mixtures. Peptides in

the mixtures can be subjected to collision-induced dissociation (CID) to generate fragments, and the masses of the fragments can be determined. The masses of the peptide fragments are used to identify the proteins by searching databases (e.g., NCBI). The identified proteins are used as biomarkers to identify bacteria (Fenselau et al. 2007; Pribil et al. 2005; Russell et al. 2007). In contrast, top-down methods introduce intact (undigested) proteins into the mass spectrometer. The intact proteins are fragmented into smaller peptides in tandem mass spectrometry (MS/MS). The experimental MS/MS spectra are compared with in silico-generated MS/MS spectra from protein sequences in proteome databases for rapid identification of bacteria (Demirev et al. 2005; Wynne et al. 2009). Top-down methods have also been successfully applied to distinguish a pathogenic *E. coli* strain (O157:H7) from the non-pathogenic strains (non-O157:H7) (Fagerquist et al. 2010). Spectral reproducibility of bioinformatics-enabled approaches is not critical as long as the ions of biomarkers are consistent with the sequences in the database. Thus, bioinformatics-enabled approaches do not require rigorously standardized protocols across laboratories to the extent required by library-based approaches. However, bioinformatics-enabled approaches are rarely applied to microorganisms that do not have fully sequenced genomes and readily available protein/peptide databases.

Successes at the Genus and Species Levels

Genus- and species-level characterization of bacteria using MALDI-TOF MS with library-based and/or bioinformatics-enabled methods has been successfully applied in many areas. For example, in clinical microbiology, MALDI-TOF MS has been used to identify pathogens directly from monomicrobial positive blood cultures (Klein et al. 2012; Martinez et al. 2014; Rodriguez-Sanchez et al. 2014) and urine samples (Ferreira et al. 2010b; Rossello et al. 2014; Wang et al. 2013) . Correct identification rates at the genus and species levels shown in these studies range from 98% (Martinez et al. 2014) down to 70% (Klein et al. 2012). Gram-negative bacteria have been reported to be more readily identified correctly than Gram-positive bacteria (Klein et al. 2012). Schrottner et al. (2014) reported that MALDI-TOF MS can distinguish between two opportunistic pathogens, *Myroides odoratus and Myroides odoratiminus* at the species level, and results were comparable to those obtained with 16S rDNA sequencing. Zhang et al. (2014b) used MALDI-TOF MS to identify *Lactobacilli* isolated from saliva samples of adults with dental caries. Results showed that 88.6% of *Lactobacillis* isolates and 95.5% of *non-Lactobacillis* isolates were correctly identified at the genus level using MALDI-TOF MS. These rates were comparable to those obtained using 16S rDNA sequencing (Zhang et al. 2014b). Hsueh et al. (2014) showed that *Acinetobacter* species isolated from blood samples could be correctly identified using MALDI with commercially available software (Bruker's Biotyper). The correct identification rate for various *Acinetobacter* species ranged from 98.6% down to 72.4% (Hsueh et al. 2014). Both library-based (Ilina et al. 2010) and bioinformatics-enabled methods (Xiao et al.

2014c) have been successfully applied to identify the etiologic agent of stomach ulcers, *Helicobacter pylori*, a Gram-negative, microaerophilic bacterium with high genetic variability. Furthermore, differentiation between *Streptococcus pneumonia* and some closely related species, such as, *Streptococcus pseudopneumoniae*, *Streptococcus mitis* and *Streptococcus ordis*, is difficult and misidentifications occur with routinely employed molecular methods (Werno et al. 2012). MALDI-TOF MS has been reported to facilitate identification of 75 % of *Streptococcus* isolates at the genus and species levels (Wessels et al. 2012). Werno et al. (2012) suggest that rigorous examination of the mass peak profiles can enhance the ability of MALDI-TOF MS to distinguish *Streptococcus pneumonia* from nonpneumococcal isolates . MALDI-TOF MS is not only applicable to aerobic bacteria, but also applied to anaerobic bacteria. Zarate et al. (2014) used MALDI-TOF MS to identify 106 clinical isolates of anaerobic bacteria. The correct identification rate at the genus and species levels was 95.3 %, comparable to that obtained using conventional biochemical tests . The possibility of using MALDI to identify and type anaerobic bacteria has been reviewed recently (Nagy 2014).

Recently, MALDI-TOF MS has been employed in food microbiology. *Enterococcus* species are considered to be secondary contaminants of food and often play roles in food spoilage. Some closely related enterococcal species are difficult to discriminate using 16S rDNA sequencing, while one study has shown that *Enterococcus* can be rapidly identified at the species level using MALDI-TOF MS (Quintela-Baluja et al. 2013). Other food-borne pathogens and spoilage bacteria, such as *Arcobacter* spp., *Helicobacter* spp., *Campylobacter* spp., *Lactobacillus* spp., *Pediococcus* spp., *Leuconostoc* spp., *Streptococcus* spp., *Clostridium* spp., and *Staphylococcus* spp., have also been identified at the species level using library-based MALDI-TOF MS methods (Alispahic et al. 2010 Bohme et al. 2011a; Bohme et al. 2011b; Han et al. 2014; Kern et al. 2013; Regecova et al. 2014; Wieme et al. 2014b). Some *Bacillus* species are pathogens or spoilage agents in food products. MALDI-TOF MS was shown to be more effective than 16S rDNA sequencing to differentiate *Bacillus subtilis* and *Bacillus cereus* from *Bacillus amyloliquefaciens* and *Bacillus thuringiensis* (Fernandez-No et al. 2013). Acetic acid bacteria are involved in the industrial production of vinegar. Andrés-Barrao et al. (2013) characterized 64 strains of acetic acid bacteria belonging to the genera *Acetobacter*, *Gluconobacter,* and *Gluconacetobacter* using MALDI-TOF MS with the SARAMIS™ software package (Spectral Archive and Microbial Identification System; Anagnostec Gmbh, Germany). Results showed that these acetic acid bacteria could be rapidly and reliably identified using fingerprint mass spectra (Andres-Barrao et al. 2013). Bohme et al. (2013) used two technologies, 16S rDNA sequencing and MALDI-TOF MS, to identify pathogens in seafood. DNA sequencing identified only 50 % of the strains at the species level and performed relatively poorly with regard to identification of members of the *Pseudomonas* and *Bacillus* genera. In contrast, MALDI-TOF MS facilitated correct identification of 76 % of the isolated strains and showed a higher rate of correct classification of members of the *Pseudomonas* and *Bacillus* genera.

In addition to the applications in clinical and food microbiology, MALDI-TOF MS has been used to characterize culturable bacterial populations isolated from various environments, including laboratory mice and rats (Goto et al. 2012), horse semen samples (Masarikova et al. 2014), human feces (Samb-Ba et al. 2014), air (Setlhare et al. 2014), as well as recombinant bacteria (Xiao et al. 2014a). All of these studies suggest that MALDI-TOF MS is a rapid, reliable, and alternative method for characterizing bacteria particularly at the genus and species levels.

Strain-Level Characterization: Successes, Challenges, and Strategies

MALDI-TOF MS has shown promise at the strain level. For example, Christner et al. (2014) used MALDI-TOF MS to type 294 *E. coli* isolates collected during a large outbreak in northern Germany. Strain-specific biomarker peaks were reported, and 99 % of the *E. coli* strains were correctly identified using strain-specific biomarkers (Christner et al. 2014). Similarly, Schafer et al. (2014) used MALDI to identify ERIC-genotypes of *Paenibacillus larvae* strains. *P. larvae* is the causative agent of American foulbrood disease in honeybees. Results showed that with a reference database, ERIC I and II types of *P. larvae* strains could be unambiguously identified. In the food industry, *Lactobacillus brevis* strains exhibit varying beer-spoiling abilities. Kern et al. (2014) showed that strain-level identification of 17 *Lactobacillus brevis* strains was achieved in 90 % of 204 spectra (Kern et al. 2014a).

The MALDI-TOF MS fingerprint technique also shows promise in tracking strains isolated from different environmental sources. Siegrist et al. (2007) showed that a limited number of environmental *E. coli* strains could be grouped according to the source from which they were isolated. Similarly, Niyompanich et al. (2014) showed that 6 out of 11 clinical and environmental *Burkholderia pseudomallei* strains were grouped correctly according to their respective sources . Strain-level characterization has also been demonstrated for *Propionibacterium acnes* (Nagy et al. 2013) and *Bacteroides fragilius* (Nagy et al. 2011). Fujinami et al. (2011) reported using MALDI-TOF MS to successfully discriminate 23 *Legionella pneumophila* strains . In addition, MALDI-TOF MS has been used to detect antibiotic resistance associated with identified strains, which is extensively reviewed elsewhere in this book (Chapter(s) XX).

While successes using MALDI-TOF at the strain level have been described, characterization at this level faces many more challenges than applications at higher taxonomic ranks. For example, Zeller-Peronnet et al. (2013) studied the discriminatory power of MALDI-TOF MS to differentiate 24 strains within *Leuconostoc mesenteroides* and *Leuconostoc pseudomesenteroides* species. Results showed that, although individual species could be readily identified, only half of the strains could be correctly identified to the strain level, suggesting that the discriminatory power of MALDI might not be adequate for characterization of these two species at the strain level (Zeller-Peronnet et al. 2013). Several studies have explored whether MALDI-

TOF MS can differentiate MRSA from methicillin-sensitive *Staphylococcus aureus* ones (MSSA), but there is limited consensus (Bernardo et al. 2002; Jackson et al. 2005; Lasch et al. 2014; Majcherczyk et al. 2006; Walker et al. 2002). Conflicting results may be because of low quality and reproducibility of the fingerprint mass spectra. In addition, cultivation conditions and sample preparation methods have also been suggested to affect strain-level differentiation (Goldstein et al. 2013; Balážová et al. 2014a). Besides *Staphylococcus aureus*, MALDI-TOF MS has also showed insufficient discriminatory power for typing strains of other bacteria. Lasch et al. (2014) reported that MALDI could not reliably differentiate *Enterococcus faecium* strains based on clonal complexes and multilocus sequence types. Kern et al. (2014) reported that when highly similar strains of the beer-spoilage bacterium *Pectinatus frisingensis* were incorporated for analysis using MALDI, the correct identification rate for *P. frisingensis* at the strain level decreased from 73 % (using a relatively diverse set of strains) to 60 % (including those that were highly similar to one another; Kern et al. 2014b) . A more comprehensive review of reports on bacterial strain categorization, differentiation, and identification using MALDI-TOF MS is available (Sandrin et al. 2013).

Commercially Available Software

In library-based approaches, bacterial characterization requires comparison of mass spectra of unknowns with those of reference spectra of known bacteria. Though visual inspection can sometimes provide a qualitative assessment of the similarity between mass spectra at the genus and possibly the species levels, software algorithms have been developed to provide more objective and quantitative assessments. Such tools are critically important at the strain level, where spectra of closely related strains are often extraordinarily similar, and reliable discrimination requires sensitive and repeatable measures. Many software solutions, such as custom R packages, Microsoft Excel (Microsoft Corporation, Redmond, WA), MATLAB (MathWorks, Matick, MA), and BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium), have been applied to enhance analysis (Croxatto et al. 2012; Pavlovic et al. 2013; Sandrin et al. 2013).

Currently, two of the most frequently used commercially available software packages are BioTyper (Bruker Daltonics, Billerica, MA, USA) and SARAMIS (bioMérieux, SA, Marcyl'Etoile, France). BioTyper is commonly used in the clinical setting with the MicroFlex LT mass spectrometer (Bruker Daltonics; Billerica MA, USA). SARAMIS is offered by bioMérieux and is routinely used with the Vitek MS system (bioMérieux SA; Marcyl'Etoile, France). Both BioTyper and SARAMIS provide a database environment and spectrum comparison algorithm that allows for quantitative comparison and identification of bacteria within the database. These two software packages also allow users to build custom databases, in which spectra acquired in-house can be added to the database. For quantitative comparison, both BioTyper and SARAMIS require summarization of mass spectra

of biological and/or technical replicates of bacteria. The resulting composite mass spectra are called main spectral projections (MSPs) in BioTyper and a SuperSpectrum™ in SARAMIS.

BioTyper and SARAMIS have been most commonly used to identify bacteria at the genus and species levels. BioTyper uses a score-based classification system for bacterial identification, while SARAMIS uses a percentage-based method. These algorithms are described more fully later in this chapter. In addition, studies have suggested that BioTyper has similar (Mather et al. 2014) or superior (Chen et al. 2013) abilities to SARAMIS with regard to identification at the genus and species levels. With regard to the strain-level characterization, BioTyper has been suggested to be useful in characterizing bacteria at the strain level, but typically with the assistance of additional software, such as ClinProTools (Bruker Daltonics) and R. Ayyadurai et al. (2010) reported that, with assistance of ClinProTools, three biotypes of *Yersinia pestis* strains could be differentiated. Nakano et al. (2014) used three ClinProTools models to differentiate vanA-positive *Enterococcus faecium* from vanA-negative *Enterococcus faecium* with MALDI and BioTyper. All three ClinProTools models yielded > 90% recognition capability . Karger et al. (2011) used BioTyper with the R-package caMassClass to filter out some peaks, and this data-reduction strategy enhanced categorization of strains of Shiga toxin-producing *E.coli* . Strain-level applications of SARAMIS have not been frequently reported in the literature.

Assessing Strain-Level Performance

Objectives

Strain-level characterization often entails one or more three distinct objectives: (1) strain categorization, (2) strain differentiation, and (3) strain identification (Fig. 6.4). These three objectives often require different levels of taxonomic resolution. Strain categorization involves grouping bacterial strains that share a particular trait, such as their origin (Dubois et al. 2010; Siegrist et al. 2007), antibiotic resistance (Shah et al. 2011; Wolters et al. 2011), pathogenicity (Stephan et al. 2011), and/or as different subspecies/biotypes (Ayyadurai et al. 2010; Lundquist et al. 2005; Zautner et al. 2013). Strain categorization does not typically involve discriminating single strains. In contrast, strain differentiation requires distinguishing single strains and thus higher taxonomic resolution. Many studies have reported that bacterial strains, of both medical and environmental relevance, can be differentiated based on the presence and/or absence of one or more strain-specific biomarker peaks (Donohue et al. 2006; Everley et al. 2008; Ghyselinck et al. 2011; Majcherczyk et al. 2006; Ruelle et al. 2004; Zautner et al. 2013) or by cluster analysis (Balážová et al. 2014b; Holland et al. 2014a). Among these three objectives, strain identification requires the highest taxonomic resolution. When comparing with a reference library, strains often cannot be confidently identified based on the presence of only one or a few

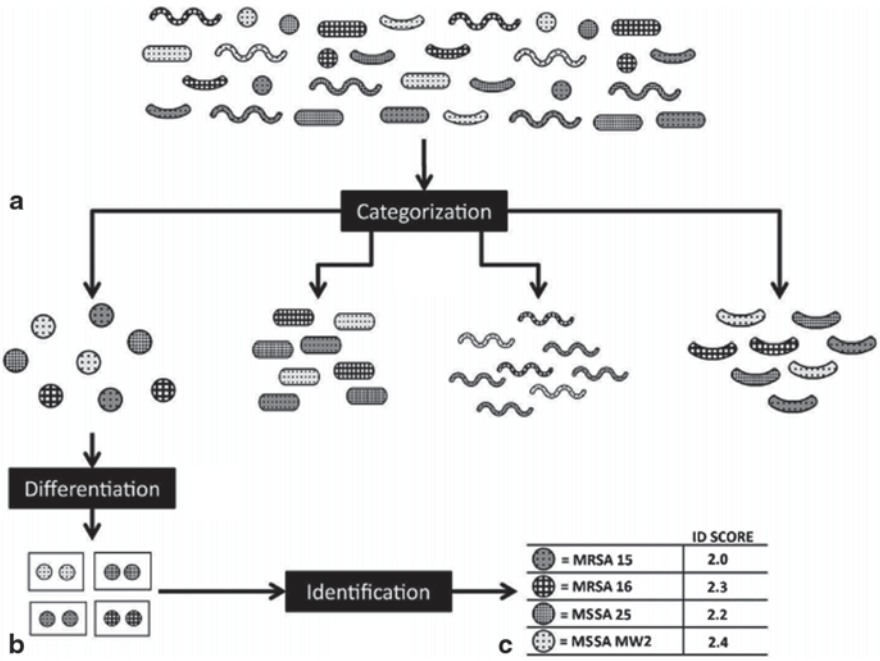

Fig. 6.4 Objectives of strain-level applications of MALDI-TOF MS-enabled characterization of bacteria have included categorization (**a**), differentiation (**b**), and identification (**c**). The requisite level of taxonomic resolution tends to increase as one progresses from efforts to categorizing strains to identifying individual strains. (Adapted from Sandrin et al. 2013, copyright John Wiley & Sons)

biomarker peaks. Analysis of the entire spectrum (Holland et al. 2014b) with rigorous analytical tools, such as those in R, ClinProTools, and BioTyper software (Nakano et al. 2014), is often required to obtain reliable strain identification.

Reproducibility

Closely related strains of bacteria yield highly similar mass spectra (Fig. 6.5). To reliably characterize strains, the reproducibility of replicated mass spectra of the same strain must be quantified before conducting further analysis. Here, reproducibility refers to how similar replicate spectra of the same strain are to one another based on comparing peak presence/absence and/or peak intensity. Reproducibility (similarity) between replicates of the same strain must exceed the similarity of mass profiles of closely related bacterial strains. Several previous studies have examined reproducibility based primarily on visual inspection of spectra (Arnold and Reilly 1998; Jackson et al. 2005), while more recently, studies have quantified reproducibility more rigorously using software packages described previously. The coefficient of

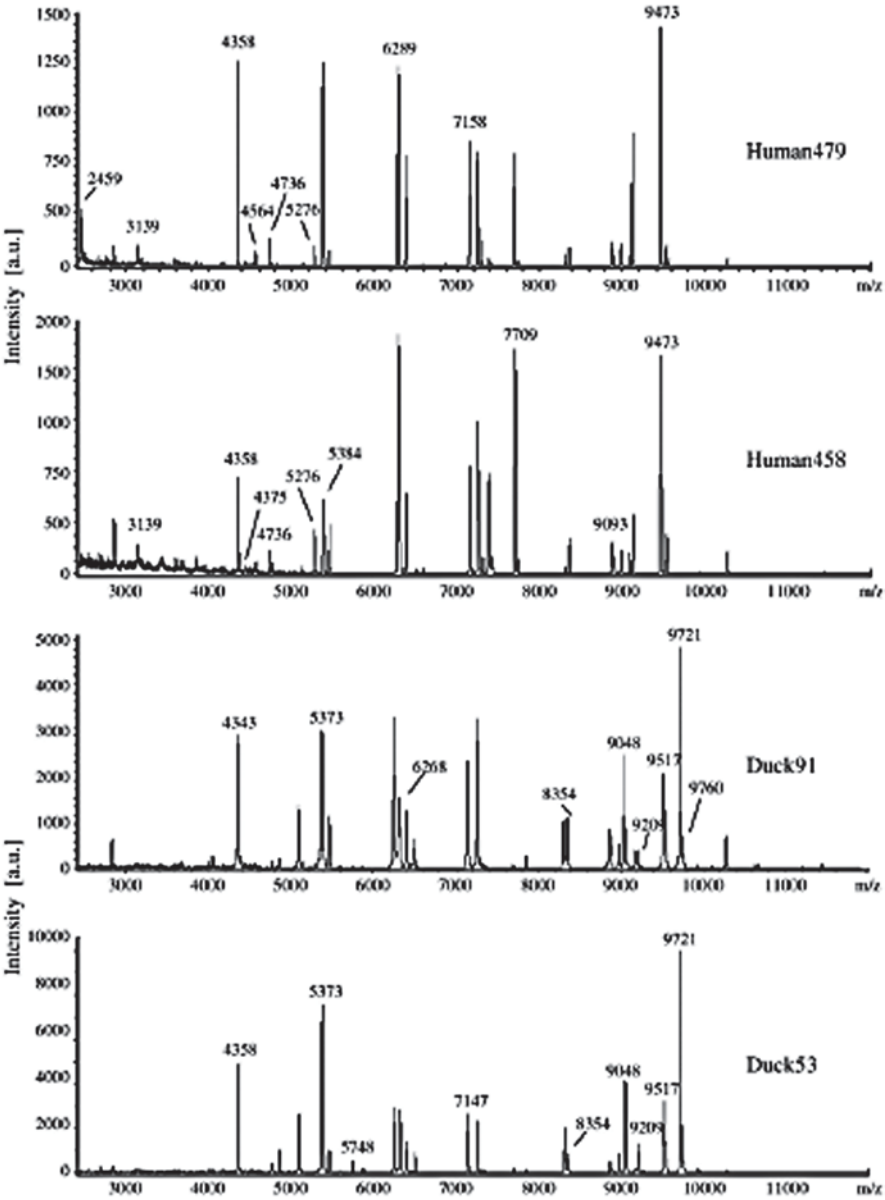

Fig. 6.5 Bacteria of the same species tend to produce similar MALDI-TOF spectra. Four environmental isolates of *Escherichia coli* yielded MALDI spectra that were particularly similar within a single environmental source (i.e., duck or human origin), but adequately distinct across different sources to allow characterization of isolates based upon source. (Adapted from Siegrist et al. 2007, with permission from Elsevier)

variation of each mass signal of replicate spectra has been used to quantify the reproducibility of replicate spectra (Freiwald and Sauer 2009). Chen et al. (2008) combined analysis of variance and principal component analysis (ANOVA–PCA) to quantify the reproducibility of replicate spectra . Toh-Boyo et al. (2012) reported using ANOVA, PCA, and multivariate ANOVA (MANOVA) to assess reproducibility . The curve-based Pearson correlation coefficient has also been used to measure reproducibility (Giebel et al. 2010; Schumaker et al. 2012; Zhang et al. 2014a). Binary coefficients (which do not include peak intensity measurements), including the Dice similarity coefficient (Ziegler et al. 2012) and Jaccard index (Erler et al. 2014), have also been used. As detailed below, both experimental and data analysis factors have been reported to affect the reproducibility of replicate spectra.

Group Separation/Performance Metrics

Score

BioTyper (Bruker Daltonics, Billerica, MA, USA) and SARAMIS (bioMérieux, SA, Marcyl'Etoile, France) are the most commonly used software and database packages for bacterial identification at the genus and species levels. Both use a score/percentage-based method to assess performance. BioTyper compares patterns of peaks of unknowns with reference spectra based on peak position, peak intensity, and peak frequencies. A log score is obtained for each comparison using a manufacturer-defined algorithm. The manufacturer proposes that a score ≥ 2 indicates species identification; a score between 1.7 and 1.9 indicates genus identification, and a score < 1.7 indicates no identification. Similar to BioTyper, SARAMIS uses a confidence percentage for genus and species identifications. Genus-level identification is acceptable when the confidence percentage ranges from 98% to 90%. Species identification is acceptable when the confidence percentage is $> 98\%$. Identification is not acceptable when the confidence percentage is $< 90\%$ (Chen et al. 2013). With regard to the Sepsityper™ kit (Bruker Daltonics; Billerica, MA, USA) to directly identify bacteria from positive blood cultures, the score cutoff is 1.8 for identification at species level and 1.6 for that at the genus level (Nonnemann et al. 2013).

Reliable identification of aerobic and anaerobic bacteria at the genus and species levels using BioTyper scores has been reported in many studies (Balada-Llasat et al. 2013; Coltella et al. 2013; Ferreira et al. 2010a; Hsueh et al. 2014; Ikryannikova et al. 2013; Lacroix et al. 2014; Mather et al. 2014; Sanchez-Juanes et al. 2013; Schulthess et al. 2014). The correct identification rate at the genus and species levels ranges from 100% (Ikryannikova et al. 2013; Sanchez-Juanes et al. 2013) down to 72% (Hsueh et al. 2014). Furthermore, some studies have suggested that reducing the species cutoff, for example, from 2.0 to 1.7 (Mather et al. 2014; Pereyre et al. 2013; Schulthess et al. 2014), and from 1.8 to 1.5 for positive blood cultures using Sepsityper™ kit (Nonnemann et al. 2013), can increase species identifica-

tion rates for some species. However, unreliable and/or no identification for some bacterial species, such as *Nocardia* spp. (except for *N. nova* and *N. otitidiscaviarum*), *Tsukamurella* spp., *Gordonia* spp., and *Avibacterium spp*. (except for *A. paragallinarum*), using BioTyper scores has also been reported (Alispahic et al. 2014; Hsueh et al. 2014).

Jackknife/Bootstrapping/Threshold Cutoffs

The jackknife test has also been used to evaluate the performance of MALDI. The principle of the jackknife test is to take out one entry from a group (e.g., genus, species, and/or strains) and to identify this entry against different groups (Johnson and Wichern 2007). The procedure is repeated for all entries in this group, and the percentage of correct group identifications is used to assess the accuracy of MALDI for characterization of the group. The jackknife test has been used to evaluate the accuracy of bacteria identification at the species (De Bruyne et al. 2011) and strain levels (Goldstein et al. 2013). Besides the jackknife statistic, bootstrapping has also been used to estimate the performance of classification models of isolates. For example, AlMasoud et al. (2014) used PCA and support vector machines (SVM) to supervise peak classification of a peak table matrix containing 243 unique peaks for characterizing *Bacillus* spp. The SVM models were validated by using a bootstrap replacement procedure coupled with cross-validation for selection of model parameters. Classification accuracies at the *Bacillus* species level reached about 90 % using the validated SVM models.

Culture Conditions

A variety of media, including solid agar (e.g., Grosse-Herrenthey et al. 2008; Pennanec et al. 2010) and liquid broth (e.g., Wensing et al. 2010), have been used to support the growth of bacteria to be characterized by MALDI. Mass spectra of bacteria consist of peaks mainly derived from ribosomal proteins and other abundant proteins (Ryzhov and Fenselau 2001; Teramoto et al. 2007b). Ribosomal proteins are highly conserved and are not expected to be affected by culture conditions (Arnold and Reilly 1999). Several studies have also shown that a core set of species-specific peaks are constantly observed regardless of the media used (De Bruyne et al. 2011; Grosse-Herrenthey et al. 2008; Hsu and Burnham 2014; Kern et al. 2013; Lartigue et al. 2009; Sauer et al. 2008); however, culture conditions can influence the expression pattern of other proteins (Valentine et al. 2005). Thus, media effects have been widely reported (Horneffer et al. 2004; Moura et al. 2008; Ruelle et al. 2004; Walker et al. 2002), though some studies have suggested that the effects are subtle and do not affect the overall ability of MALDI to characterize bacteria (Bernardo et al. 2002; Dieckmann et al. 2008; Kern et al. 2013; Vargha et al. 2006).

Taken together, these results indicate that medium effects at the strain level may be more pronounced and significant than at higher taxonomic ranks.

Medium Type

Several studies have shown that medium components can affect the rate of successful identification of many bacteria. For example, Anderson et al. (2012) reported that the correct identification rate at the genus level for bacteria grown on different types of agars varied for *Pseudomonas, Staphylococcus,* and enteric isolates. The effect was most pronounced for *Staphylococcus* isolates, varying from 75% on colistin-nalidixic acid agar to 95% on blood agar and mannitol salt agar (Anderson et al. 2012). Variations have been reported with other *Staphylococci* using different media (Rajakaruna et al. 2009; Walker et al. 2002). Similarly, results from one study suggested that changing the medium could improve differentiation between closely related members of the family *Enterobacteriaceae* (Keys et al. 2004). Ford and Burnham (2013) grew 24 enteric Gram-negative bacteria (EGNB) and 25 non-glucose-fermenting/fastidious Gram-negative bacteria (NFGNB) on different types of agars. Results showed that the rate of successful identification on EGNB at the species level was approximately 20% less than on other types of agars. For NFGNB, rates of correct identification at the species level varied from less than 60% on OFPBL agar to 90% on sheep blood agar, and one misidentification was observed for bacteria grown on MacConkey agar (Ford and Burnham 2013).

With regard to subspecies and strain-level characterization, a few studies have reported that medium components do not affect the strain-level resolution (Bernardo et al. 2002; Dieckmann et al. 2008; Vargha et al. 2006). However, other studies have reported that medium composition significantly affects strain-level performance. Šedo et al. (2013) anaerobically cultivated strains from six *Lactobacillus* species in two kinds of liquid media, De Man-Rogosa-Sharpe broth and anaerobe basal broth, and on blood agar plates. No obvious effect of medium type on species-level resolution was observed, but some closely related strains could be distinguished only with a specific cultivation medium. Wieme et al. (2014a) studied effects of five different culture media on the differentiation of 25 strains of acetic acid bacteria, including *Acetobacter, Gluconacetobacter,* and *Gluconobacter* strains, at the species and strain levels. For each strain grown on a particular medium type, a single composite mass spectrum was obtained. Results showed that culture media did not affect species-level differentiation, but strongly affected the number of shared strain-specific peak classes in the composite mass spectra of the same strain grown on different media, in particular when the culture media did not sustain optimal growth. Balážová et al. (2014) tested the effects of four culture media on the discriminatory power of MALDI to characterize 10 strains belonging to *Mycobacterium phlei* and *Mycobacterium smegmatis* (Balážová et al. 2014a). The successful identification rate calculated from Biotyper score cutoffs was similar for all the four media at the genus level, but twofold higher for one preferable medium over the others at the species level (Balážová et al. 2014a).

Medium type has also affected strain-level differentiation and identification. The influence seems specific for particular species and strains. For example, Šedo et al. (2013) examined the influence of growth conditions on strain differentiation within the *Lactobacillus acidophilus* group (17 strains representing six different species from the *Lactobacillus acidophilus* group). Results showed that two *Lactobacillus acidophilus* strains could be distinguished after cultivation on blood agar, but could not be distinguished when grown on other kinds of media, while other *Lactobacillus acidophilus* stains could be distinguished regardless of medium types. Balážová et al. (2014) reported that, generally, M7H9 medium generated a higher correct identification rate than Herrold's egg yolk medium (HEYM) medium for characterizing *Mycobacterium* strains. The effect was even more significant for *Mycobacterium phlei* strains. For example, 89% of *Mycobacterium phlei* strains were correctly identified using M7H9 medium, while only 50% of *Mycobacterium phlei* strains were correctly identified using HEYM medium. With regard to *Mycobacterium smegmatis* strains, HEYM medium showed a lightly better identification result (60%) than M7H9 medium (52%; Balážová et al. 2014a). These studies clearly showed that medium type has the potential to affect MALDI-TOF MS profiles, but the effects may be bacterium specific. To maximize taxonomic resolution, especially at the strain level, the potential effects should be thoroughly investigated. Databases containing multiple reference strains grown on different culture media may need to be established.

Medium Form (Broth/Agar)

Besides the components in the medium, medium form (agar or broth) has also been investigated with regard to potential effects on the taxonomic resolution of MALDI-TOF MS. At the species level, Lotz et al. (2010) cultivated 311 *Mycobacterium* strains both on agar plate and in liquid broth. Using an intact cell preparation method, correct identification rates were 97% for solid media and only 77% for liquid media. The low identification rate for liquid media was suggested to be because of the failures of spectrum acquisition (Lotz et al. 2010). Balada-Llasat et al. (2013) cultured 178 mycobacterial isolates using both solid and liquid media. Using a protein extraction-based sample preparation method, 93.8% of the isolates were identified correctly at the species level with both forms of media, suggesting that medium forms may not affect MALDI resolution at the species level. At the strain level, though, Goldstein et al. (2013) cultured MRSA and MSSA on both solid agar plates and broth media. Results showed that liquid media generated higher jackknife values when differentiating MRSA from MSSA, suggesting that culture in liquid media enhances the discriminatory power of MALDI. The higher discriminatory power may be because of the more homogeneous populations of cells synchronized in their growth phase in the broth cultures, whereas on an agar plate, colonies consist of older, senescent cells in the center and newer, more actively growing cells at the perimeter (Sandrin et al. 2013) .

Sample Preparation

As comprehensively reviewed by Šedo et al. (2011), a myriad of diverse sample preparation techniques have been used to profile bacteria with MALDI. These diverse techniques can be classified into two types: intact cell-based and protein extract-based methods. Intact cell-based methods involve deposition of cells or cell suspensions onto the MALDI target, while protein extract-based methods involve deposition of cell extracts onto the MALDI target. While intact cell-based methods do not involve intentional extraction of cell materials, the chemicals added may still cause cell degradation. Intact-cell based methods are simpler and more rapid than cell protein extract-based methods, because they do not require additional steps to break cells and extract proteins (Sauer et al. 2008). Sample preparation methods have been suggested to affect the taxonomic resolution of MALDI-based approaches to bacterial characterization (Šedo et al. 2011). For example, Zampieri et al. (2013) used both intact cell-based and protein extraction-based methods to identify 11 bacteria isolated from bovine semen at the genus and species levels. The intact cell-based method resulted in correct identification of nine bacteria at the genus and species levels, while a protein extraction-based method afforded correct identification of all 11 bacteria at the genus and species levels (Zampieri et al. 2013). Therefore, many studies have explored multiple preparation methods to maximize the taxonomic resolution of MALDI profiling (e.g., Dieckmann et al. 2008; Ruelle et al. 2004; Williams et al. 2003). Interestingly, commercially available platforms have been applied using different sample preparation approaches. For example, Lohmann et al. (2013) used a cell extract-based sample preparation approach with Bruker's BioTyper, but an intact cell-based approach with bioMérieux's SARAMIS.

Intact Cells

Generally, cells have been directly deposited onto MALDI targets in two ways. Some studies have described placement of cell suspensions/colonies directly on the MALDI targets and subsequently overlaid matrix solutions which usually contain TFA and ACN (Carbonnelle et al. 2007; Christner et al. 2014; Han et al. 2014; Jackson et al. 2005; Walker et al. 2002). Other studies have reported mixing cell suspensions with matrix solutions prior to deposition onto the MALDI target (AlMasoud et al. 2014; Arnold and Reilly 1998; Dickinson et al. 2004; Donohue et al. 2006; Helmel et al. 2014; Moura et al. 2008; Ryzhov et al. 2000; Welham et al. 1998). Both "overlaid" and "premixed" approaches of intact cell-based sample preparation have afforded strain-level resolution (Kuehl et al. 2011; Zautner et al. 2013); however, only a few studies have directly compared the effects of these two deposition approaches on the taxonomic resolution of MALDI profiling of bacteria. Jackson et al. (2005) compared the performance of MALDI to profile MRSA at the

stain level by directly depositing MRSA colonies from agar plates with directly depositing MRSA cell suspensions onto the target plate. Results indicated that directly deposited colonies yielded higher quality spectra and higher reproducibility than deposited cell suspensions (Jackson et al. 2005). Kuehl et al. (2011) applied three techniques, including premixed, overlaid (ethanol added as an organic modifier), and a sandwich method with cells placed between two layers of matrix, for applying samples to the MALDI target. Results showed that the sandwich method generated the highest quality mass spectra when characterizing *Enterococcus faecalis* (Kuehl et al. 2011). Toh-Boyo et al. (2012) compared the reproducibility of the mass spectra resulting from laser sampling at different regions within a single target well (intrasample) and between target wells (intersample) using both overlaid and premixed pipet-based deposition methods with three different matrices . The authors observed that the crystalline morphology of the sample on the target greatly influenced intrasample reproducibility (Fig. 6.6a). Samples deposited using the pipet-based premixed method yielded less variability between spots for a single sample than the pipet-based overlay method (Fig. 6.6b). More recently, AlMasoud et al. (2014) reported similar results showing that a premixed deposition method worked best for typing *Bacillus* and *Brevibacillus* species over the other three deposition methods, including (1) overlaying matrix onto protein extracts, (2) overlaying protein extracts onto matrix, and (3) a sandwich method in which protein extracts were situated between two layers of matrix .

Protein Extracts

Similar to intact cell-based methods, several protocols have been described to extract proteins from cells. The most commonly used protein extraction method is an ethanol-formic extraction, in which a crude protein extraction is performed in a microcentrifuge tube (e.g., Freiwald and Sauer 2009). In addition, plate-based formic acid extraction has also been described (e.g., Schulthess et al. 2014).

Several studies have suggested that protein extraction-based methods afford higher taxonomic resolution than intact cell-based methods. For example, Schulthess et al. (2014) compared three sample preparation methods for identification of 190 Gram-positive rods including 64 species from 21 genera. Results showed that species-level identification rates were higher with a plate-based formic acid extraction and an ethanol-formic acid extraction than with an intact cell-based direct smear method. Rates of correct identification were 68.4 % with plate-based formic acid extraction, 71.6 % with ethanol-formic acid extraction, and 63.7 % with a direct smear method.

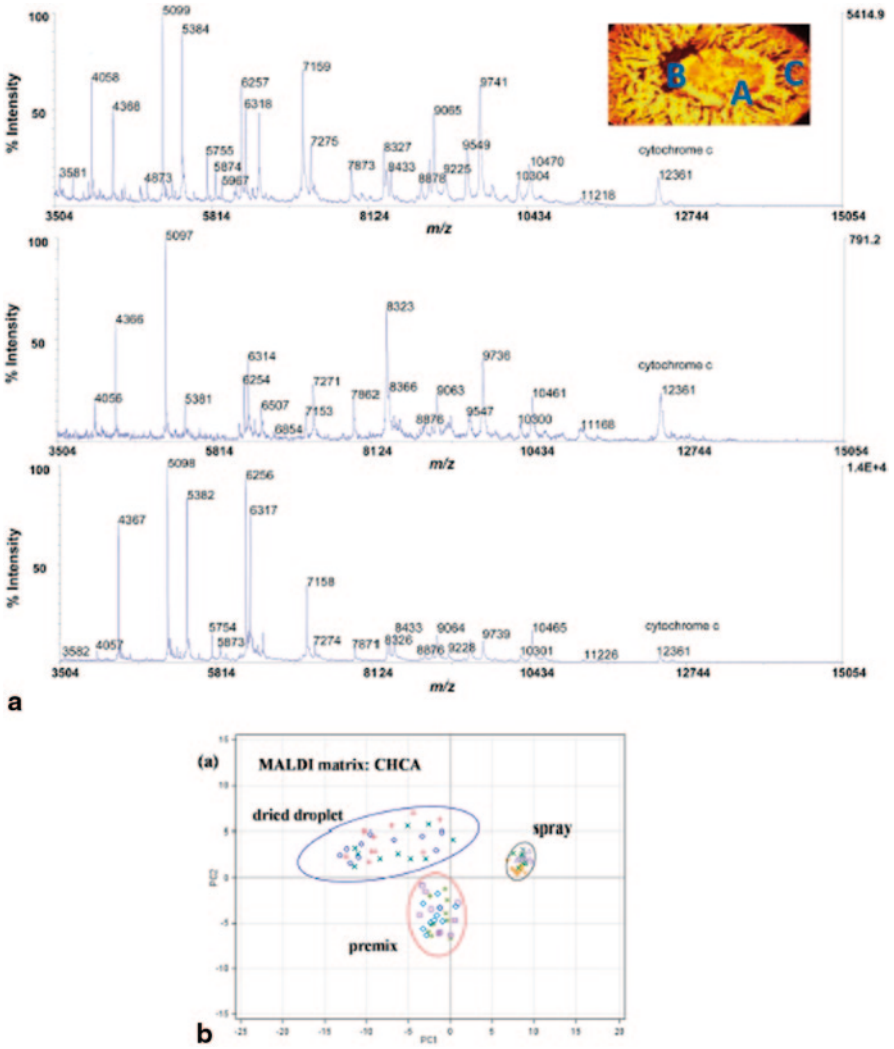

Fig. 6.6 MALDI-TOF spectra from a single sample in a single MALDI target well yielded distinct spectra (**a**). Similarly, the mode of application of sample to the MALDI target plate affected spectra (**b**). These findings underscore the need to (i) ensure sample preparation techniques maximize sample homogeneity on the target plate and (ii) ensure standardized sample deposition protocols are followed when using library-based MALDI-TOF-based approaches at the strain level. (Adapted from Toh-Boyo et al. 2012, copyright American Chemical Society)

Pretreatment to Enhance Taxonomic Resolution

Several pretreatments have been used to increase the discriminatory power of library-based MALDI fingerprint approaches. Horneffer et al. (2004) used wet-heat treatment to extract additional analytes that facilitated strain-level resolution of *B.*

subtilis and *B. cereus*. Enzymatic pretreatment has also been used to facilitate more complete extraction of cell contents. Two of the most commonly used enzymes are trypsin (Balážová et al. 2014b; Krasny et al. 2014; Krishnamurthy et al. 1996) and lysozyme (Giebel et al. 2008; Vargha et al. 2006). Balážová et al. (2014) used trypsin to digest cells of *S. aureus, Staphylococcus haemolyticus,* and *B. subtilis* and applied a 2-min microwave irradiation after digestion. Strain-level differentiation was achieved for *S. aureus* and *S. haemolyticus*, and improved for *B. subtilis* based on ecotypes. An increase in the number of strain-specific peaks was also observed when using this microwave-assisted tryptic digestion sample preparation method (Balážová et al. 2014b). Abdelhamid et al. (2014) used a preconcentration technique, ultrasound-enhanced surfactant-assisted dispersive liquid–liquid microextraction (UESA-DLLME) technique, with *Pseudomonas aeruginosa* and *S. aureus*. This method improved the number of biomarker peaks and identification. Nanoparticles, for example, zinc oxide nanoparticles modified with polymethyl methacrylate, have also been synthesized for extracting bacteria from aqueous samples, which enhanced the sensitivity and quality of MALDI-MS spectra for characterizing bacteria such as *S. aureus* and *P. aeruginosa* (Gedda et al. 2014). Modification of the surface of the MALDI target has also been applied to enhance characterization of bacteria. For example, Hasan et al. (2014) and Gopal et al. (2013) demonstrated that using titanium chips as MALDI target and with appropriate surface pretreatments (using heat treatment at different temperatures), the chips could selectively capture either *P. aeruginosa* or *S. aureus*, leading to an improvement in spectrum quality for these two bacteria. Such modifications might be applied in the future to enhance strain-level characterization.

Data Acquisition

Modern MALDI TOF mass spectrometers can be operated with software that facilitates completely the automatic acquisition of spectra. User-defined parameters influence laser power, peak evaluation strategies, mass spectra accumulation, and laser movement on each sample. While convenient and supportive of high throughput applications, automation has been shown to affect performance. In particular, the mode of data acquisition (i.e., automated (Eddabra et al. 2012; Khot et al. 2012) or manual (Khot et al. 2012; Schumaker et al. 2012) data acquisition) may affect the taxonomic resolution of MALDI-TOF MS profiling technique. Schumaker et al. (2012) rigorously examined the effects of data acquisition modes on spectrum quality and reproducibility. Results suggested that manual data acquisition yielded more reproducible and higher quality mass spectra. Similar results were reported by Balážová et al. (2014).

Though manual data acquisition yielded more reproducible spectra, automated data acquisition is still desirable when there is a large quantity of analyses to perform, especially in clinical labs. Recent work in our lab showed that automated data acquisition can be optimized to yield spectra with reproducibility comparable to

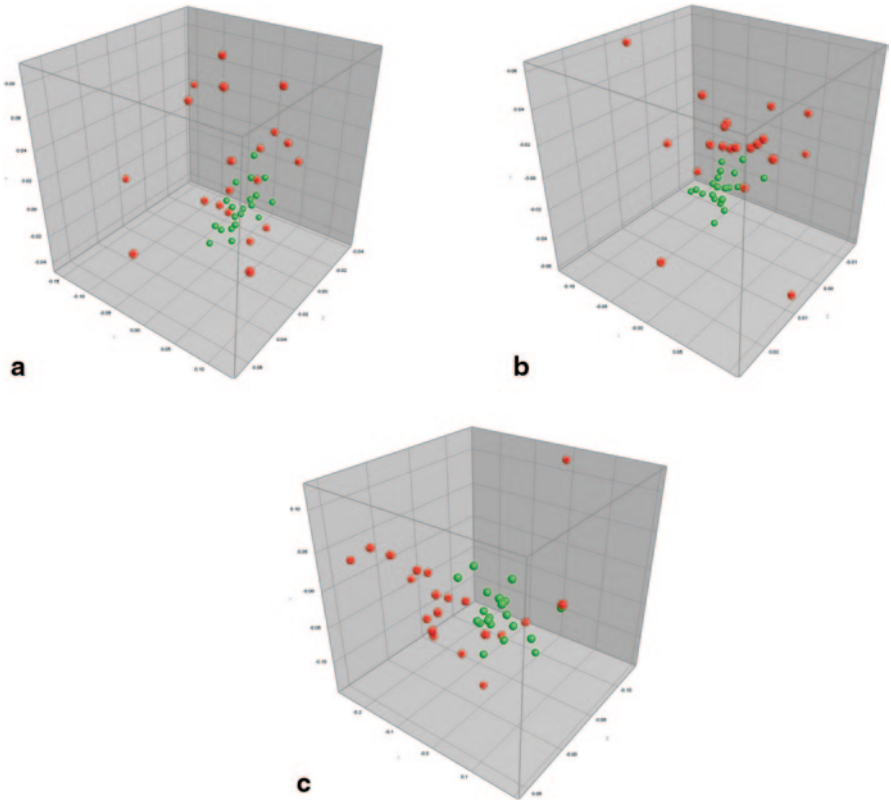

Fig. 6.7 A designed experiments approach to optimization of automated acquisition of MALDI spectra increased the reproducibility of spectra obtained from *Pseudomonas aeruginosa* (**a**), *Klebsiella pneumoniae* (**b**), and *Serratia marcescens* (**c**). (Adapted from Zhang et al. 2014a, creative commons attribution PLOS)

those obtained manually using a statistical design of experiments approach (Zhang et al. 2014a). Results showed that the reproducibility of replicate *P. aeruginosa* spectra increased from 90% to 97% by optimizing the automated data acquisition conditions. Similar results were reported for *Klebsiella pneumonia* (94% before optimization vs. 98% after optimization) and *Serratia marcescens* (85% before optimization vs. 94% after optimization; Zhang et al. 2014a) (Fig. 6.7).

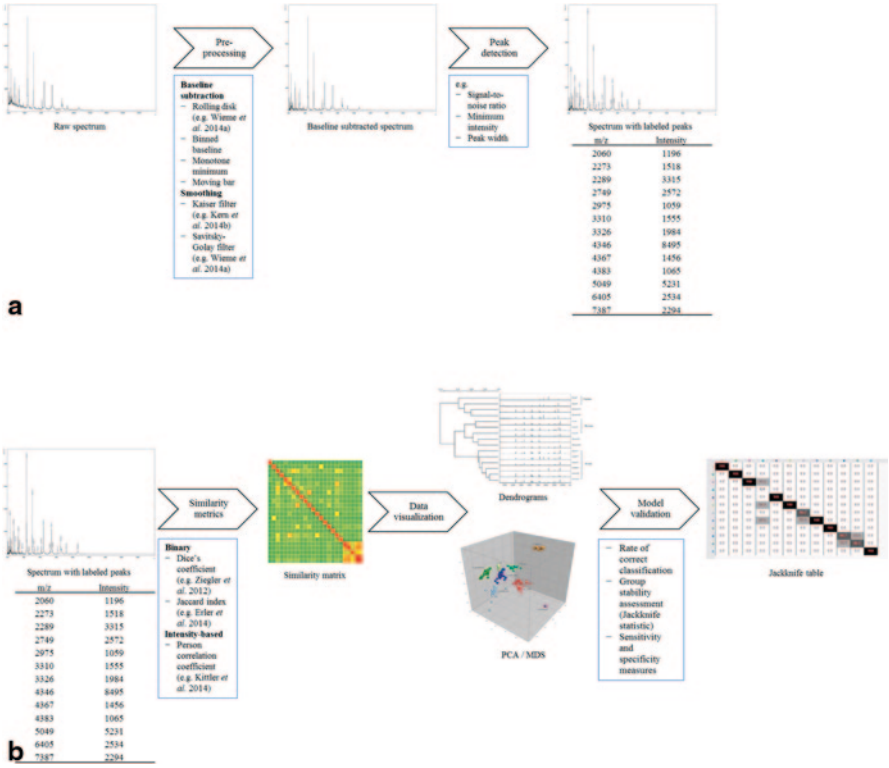

Fig. 6.8 Raw MALDI spectra are typically subjected to multiple data preprocessing (**a**) as well as postprocessing and model validation (**b**) steps. No standard workflow has been widely adopted, in spite of the fact that modifications to many steps may affect performance of the method, particularly when applied at the strain level

Data Analysis

Generally, the workflow to analyze bacteria using MALDI-TOF MS includes preprocessing, postprocessing, and model validation (Fig. 6.8). The objective of preprocessing is to reduce noise in the spectra. A variety of software has been used to preprocess raw mass spectra, such as FlexAnalysis (Bruker Daltonik, GmbH, Germany; Schrodl et al. 2012), BioNumerics (Applied Maths; Kittler et al. 2014; Wieme et al. 2014a), and DataExplorer software (Applied Biosystems, Foster City, CA, USA; Vanlaere et al. 2008).

Three common steps in the preprocessing procedures are baseline subtraction, smoothing, and peak detection. For each step, algorithms as well as parameter values associated with each algorithm can be varied. For example, baseline subtraction is used to flatten the varying base profile of a spectrum. When using BioNumerics to preprocess data, baseline subtraction can be conducted using a rolling disk algorithm (Wieme et al. 2014a) with a user-specified radius of the disk. Other baseline subtraction algorithms include monotone minimum, moving bar, and binning.

Smoothing is used to average data points with neighboring points as in a time-series of data to further reduce noise. For smoothing, Savitsky–Golay filters (Wieme et al. 2014a) and Kaiser filters (Kern et al. 2014b) have been used. Peak detection is used to separate real peaks from false peaks representing noise. A user-defined signal-to-noise ratio is usually applied for software to automatically pick peaks. Smaller signal-to-noise ratios may support higher taxonomic resolution when using peak-picking software which requires the user to specify a minimum signal-to-noise ratio such as in BioNumerics (*Applied Maths*; personal communication).

Postprocessing procedures include calculation of similarity coefficients to establish a set of classes (clusters). In the literature, similarity coefficients of spectra have been calculated in mainly two ways: using binary- and intensity-based measurements. Binary-based analysis of similarity considers only the presence/absence of peaks without considering their intensities. Such measurements can be achieved by calculating Jaccard index (Erler et al. 2014) or the Dice coefficient (Hazen et al. 2009). In contrast, intensity-based coefficients of similarity consider both the presence/absence of peaks and the peak intensity. The Pearson correlation coefficient (Kittler et al. 2014) has been used for intensity-based analysis. With regard to the dendrogram type, the unweighted pair group method with arithmetic mean (UPGMA), average linkage method (Quintela-Baluja et al. 2013), and single-link agglomerative algorithm (Andres-Barrao et al. 2013) have been used in MALDI-fingerprint studies. Though various algorithms have been applied to pre- and postprocessing of mass spectra, few studies have directly compared the effects of these algorithms on taxonomic resolution. Model validation is used to further evaluate MALDI performance on group (e.g., genus, species, and strain) separation. For example, jackknife analysis which reports the percentage of correct and false identifications has been used to quantify the stability of groups of fingerprints of MSSA and MRSA (Goldstein et al. 2013). Receiver operating characteristic (ROC) curves, which illustrates the performance of a binary classification test, were also used in the literature to evaluate *E. coli* isolate classification by spectrum similarity (Christner et al. 2014). In addition, sensitivity (true positive rate) and specificity (true negative rate) measures, which are also used for validation of the performance of binary classifications, have been reported (De Bruyne et al. 2011).

Summary

MALDI-TOF MS, shown to be a highly effective tool to characterize bacteria at the MALDI BioTyper CA System, was recently (November 2013) approved by the US FDA for the identification of Gram-negative bacterial colonies cultured from human specimens. Besides applications in clinical laboratories for identification of Gram-negative bacteria, studies have also shown that MALDI-TOF MS fingerprint-based methods can successfully characterize Gram-positive bacteria, bacteria isolated from various environments, and, in some cases, characterize bacteria at the subspecies and strain levels. Rapid innovation and advances in this area increase

the likelihood that strain-level applications will receive similar regulatory approval in the future.

Strain-level characterization using MALDI-TOF MS has included three objectives: strain categorization, strain differentiation, and strain identification. The taxonomic resolution reported for each objective has varied considerably. Generally, strain identification requires the highest taxonomic resolution, while strain categorization requires relatively low resolution. Factors, such as culture media, sample preparation method, data acquisition, and data analysis, have been shown to affect the limits of taxonomic resolution achieved. Many efforts have been made to increase taxonomic resolution. Different sample preparation methods and sample deposition have been compared with regard to their effects on taxonomic resolution. Treatments of samples, such as using enzymes to help break cell walls or using microwave radiation to help extract proteins, have also been employed. In addition, several novel approaches have been developed to increase the taxonomic resolution of MALDI-TOF MS fingerprint-based methods, including stable isotope-dependent methods that are described in more detail elsewhere in this book.

Overall, MALDI-TOF profiling of bacteria has shown remarkable promise at the genus, species, and strain levels for various bacteria; however, the limits of taxonomic resolution of this technique may impede its broader implementation. Additional efforts to maximize the taxonomic resolution of this method by optimizing experimental conditions—from culture condition through data analysis—are warranted.

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