MALDI TOF MS PROFILING OF BACTERIA AT THE STRAIN LEVEL: A REVIEW

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Since the advent of the use of matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry (TOF MS) as a tool for microbial characterization, efforts to increase the taxonomic resolution of the approach have been made. The rapidity and efficacy of the approach have suggested applications in counter-bioterrorism, prevention of food contamination, and monitoring the spread of antibiotic-resistant bacteria. Strain-level resolution has been reported with diverse bacteria, using library-based and bioinformatics-enabled approaches. Three types of characterization at the strain level have been reported: strain categorization, strain differentiation, and strain identification. Efforts to enhance the library-based approach have involved sample pre-treatment and data reduction strategies. Bioinformatics approaches have leveraged the ever-increasing amount of publicly available genomic and proteomic data to attain strain-level characterization. Bioinformatics-enabled strategies have facilitated strain characterization via intact biomarker identification, bottom-up, and top-down approaches. Rigorous quantitative and advanced statistical analyses have fostered success at the strain level with both approaches. Library-based approaches can be limited by effects of sample preparation and culture conditions on reproducibility, whereas bioinformatics-enabled approaches are typically limited to bacteria, for which genetic and/or proteomic data are available. Biological molecules other than proteins produced in strain-specific manners, including lipids and lipopeptides, might represent other avenues by which strain-level resolution might be attained. Immunological and lectin-based chemistries have shown promise to enhance sensitivity and specificity. Whereas the limits of the taxonomic resolution of MALDI TOF MS profiling of bacteria appears bacterium-specific, recent data suggest that these limits might not yet have been reached.
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I. INTRODUCTION

Recent threats to public safety posed by bioterrorism (English et al., 2003; Demirev & Fenselau, 2008a; Murray, 2010) and microbial contamination of food (Mandrell et al., 2005; Dieckmann et al., 2008; Albesharat et al., 2011) have underscored the need for rapid and reliable methods of microbial identification. Similarly, the continued emergence and rapid spread of antibiotic-resistant strains of pathogenic microorganisms, including methicillin-resistant *Staphylococcus aureus* (MRSA), emphasize the need for such rapid approaches (Camara & Hays, 2007; Hujer et al., 2009; Wolters et al., 2011). Responses to food contamination, acts of bioterrorism, and outbreaks caused by antibiotic-resistant microorganisms are influenced and limited by the methods commonly used to identify the microorganism in question. For example, epidemiological investigations rely upon identification of specific strains of microorganisms through a process commonly known as strain-typing (Tenover, Arbeit, & Goering, 1997; Welker, 2011). Bacterial strain-typing allows discrimination of pathogenic strains (e.g., *E. coli* O157:H7) from non-pathogenic ones (e.g., *E. coli* K-12). Whereas matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry (TOF MS) profiling of bacteria at the species and genus levels has been shown clearly to be rapid and effective, the utility of this approach at the strain level has not been as completely explored.

Many fundamental aspects of bacterial taxonomy, including a common definition of a bacterial species, remain highly debated (Gao & Gupta, 2012). For the purposes of this review, the term ‘‘strain’’ refers to a taxonomic level more specific and more exclusive than species. Several taxa designate classification levels below and more specific than the species level and include sub-species and serotype. Although each of these taxa convey unique meaning (e.g., serotype refers to antigenic reactions associated with a particular microorganism), this review uses the term strain to include all sub-species taxa. The focus of this review is on recent developments and the state of the science of MALDI TOF MS enabled identification of microorganisms at the strain level. Specifically, this review examines the limits of the taxonomic resolution of MALDI TOF MS bacterial profiling. Approaches to characterize bacteria via MALDI TOF MS at the species level and above are not the focus of this review because several reviews on this broader topic are available (Demirev & Fenselau, 2008b; Freiwald & Sauer, 2009; Giebel et al., 2010; Welker, 2011).

Although a host of existing methods allow rapid identification of microorganisms to the species level (and above),

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identification to the more specific "strain" taxon requires higher resolution approaches and tends to be more challenging, because strains within a single species are quite often extremely similar, genotypically and phenotypically, in spite of having different functions. At the species level, the gold standard for microbial identification remains genotypic characterization via sequencing of genes that encode the small subunit (SSU) of 16S rRNA. This approach commonly reaches the taxonomic limits of its utility at the species level and has very limited utility to resolve strains within a microbial species (Mellmann et al., 2008). Other molecular genetics approaches are commonly employed to identify and characterize strains within a microbial species (Tenover, Arbeit, & Goering, 1997). These include pulsed field gel electrophoresis (PFGE), multilocus sequence typing (MLST; Killgore et al., 2008), repetitive extragenic palindromic PCR (rep-PCR; De Bruijn, 1992), and housekeeping gene (e.g., PheS) sequence analysis (Parolo et al., 2011). Each of these approaches has been shown to have adequately high resolution to discern microbial strains from one another; however, these approaches are labor- and time-intensive techniques that might lack the requisite rapid, high-throughput nature to mitigate risks to public safety posed by acts of bioterrorism and microbial food and water contamination. Newer, more rapid approaches, including the IBIS T5000 (Ecker et al., 2008), described briefly in Section II.B.4., and the flow cytometry-based RAPID-B system (Wilkes et al., 2012), may provide very rapid, high-throughput strain-level characterization, but these approaches are not MALDI TOF MS based, and are, as such, beyond the scope of this review.

A. Overview of MALDI TOF MS Profiling of Bacteria

The evermore relevant and pressing need for rapid and reliable methods of microbial identification coupled with the substantial time and labor requirements of existing methods of microbial identification have stimulated interest in the development of alternative microbial identification methods. Mass spectrometry-enabled methods have been the subject of keen interest over the past 16 years. The rapid nature and broad applicability of mass spectrometry have instilled optimism in many that the technology has unique potential to revolutionize microbial identification. MALDI TOF MS-based approaches have been particularly popular due, in part, to the relatively simple sample preparation required and the extraordinarily rapid rate at which data can be acquired and analyzed (Fagerquist et al., 2010).

Anhalt and Fenselau (1975) employed pyrolysis-MS to pioneer use of mass spectrometry in microbial identification. Several diverse microorganisms, Gram-positive and Gram-negative, were readily distinguished with this approach. Even two representatives of the same genus, *Staphylococcus*, produced distinctive mass spectra in this seminal work. The advent of MALDI TOF MS (Karas et al., 1987) catalyzed new interest in the application of mass spectrometry to microbial identification. Several years later, Cain, Lubman, and Weber (1994) analyzed via MALDI TOF MS water soluble protein profiles of lysed bacterial cells to differentiate several diverse bacteria. Holland et al. (1996) later demonstrated that intact bacterial cells could readily be distinguished with MALDI TOF MS. The past 16 years have involved rapid development of MALDI TOF MS as a promising tool for microbial characterization of medically (Dieckmann et al., 2008; Benagli et al., 2011) and environmentally relevant (Ruelle et al., 2004; Siegrist et al., 2007; Giebel, Fredenberg, & Sandrin, 2008) bacteria. Applications have expanded even beyond the bacterial realm into fungal (Valentine et al., 2002; Kemptner et al., 2009; Marklein et al., 2009; Cassagne et al., 2011) and viral characterization (Colquhoun et al., 2006). Attempts to characterize multicellular organisms, including nematodes (Perera, Vanstone, & Jones, 2005) via MALDI TOF MS have also been described. While these successes in applying MALDI TOF MS profiling across the breadth of the microbial universe are significant, considerably less work has focused on adaptation of these methods to characterize individual strains of bacteria.

1. Library-Based Approaches

Among approaches to identify bacteria with mass spectrometry, MALDI TOF MS is most commonly used, due to its ease of use and the speed with which data can be collected. Most common, a library-based strategy is employed in which spectra of unknown bacteria are compared to libraries that contain spectra of known, reference bacteria (Fig. 1A; Tables 1–3).

As with conventional methods of microbial identification, MALDI TOF MS enabled identification nearly always requires cultivation of the microorganism of interest. Solid agar media (Grosse-Herrenthey et al., 2008; Pennanec et al., 2010) or liquid broth media (Arnold et al., 1999; Wensing, Zimmermann, & Geider, 2010) have both been used. Cultured bacterial cells are prepared for MALDI analysis. Either intact cells or cell extracts are prepared for analysis. With intact cells, MALDI matrix (e.g., ferulic acid or sinapinic acid) is mixed directly with intact cells. With cell extracts, bacterial cells are lysed via physical (e.g., sonication or bead-beating) or chemical (e.g., via exposure to TFA and formic acid/organic solvents) means to release the contents of the cells into the supernatant. The supernatant is added to matrix and analyzed with MALDI. A comprehensive review of the diverse sample-preparation methods used in bacterial profiling via MALDI is available (Šedo, Sedláček, & Zdrahal, 2011).

A variety of mass spectrometers that range from relatively low-resolution, linear detector-only benchtop units (e.g., Bruker's Microflex LT) to units with much higher resolution (e.g., Shimadzu's Axima series), can rapidly generate spectra from intact bacteria and cell extracts. Spectra are typically collected with the linear detector of MALDI TOF MS units (Hettick et al., 2006; Lartigue et al., 2009; Hahn et al., 2011), but a few investigators have used the reflector detector (available on higher resolution units) to obtain higher resolution data (Claydon et al., 1996; Bernardo et al., 2002; Vorob'eva et al., 2011). Overall, though, the resolution afforded by the linear detectors of most MALDI TOF MS units appears more than adequate to obtain useful spectra of intact bacterial cells and extracts. Spectra are typically collected from 2 to 20 kDa (Ilina et al., 2010; Ghyselinck et al., 2011), although broader (Jackson et al., 2005; Hettick et al., 2006; Teramoto et al., 2009) and narrower (Keys et al., 2004; Rajakaruna et al., 2009) ranges have both been used with success. Peaks in spectra are not typically identified in library-based approaches. While it has been hypothesized that peaks represent proteins, particularly those of ribosomal origin (Sauer and Kliem,

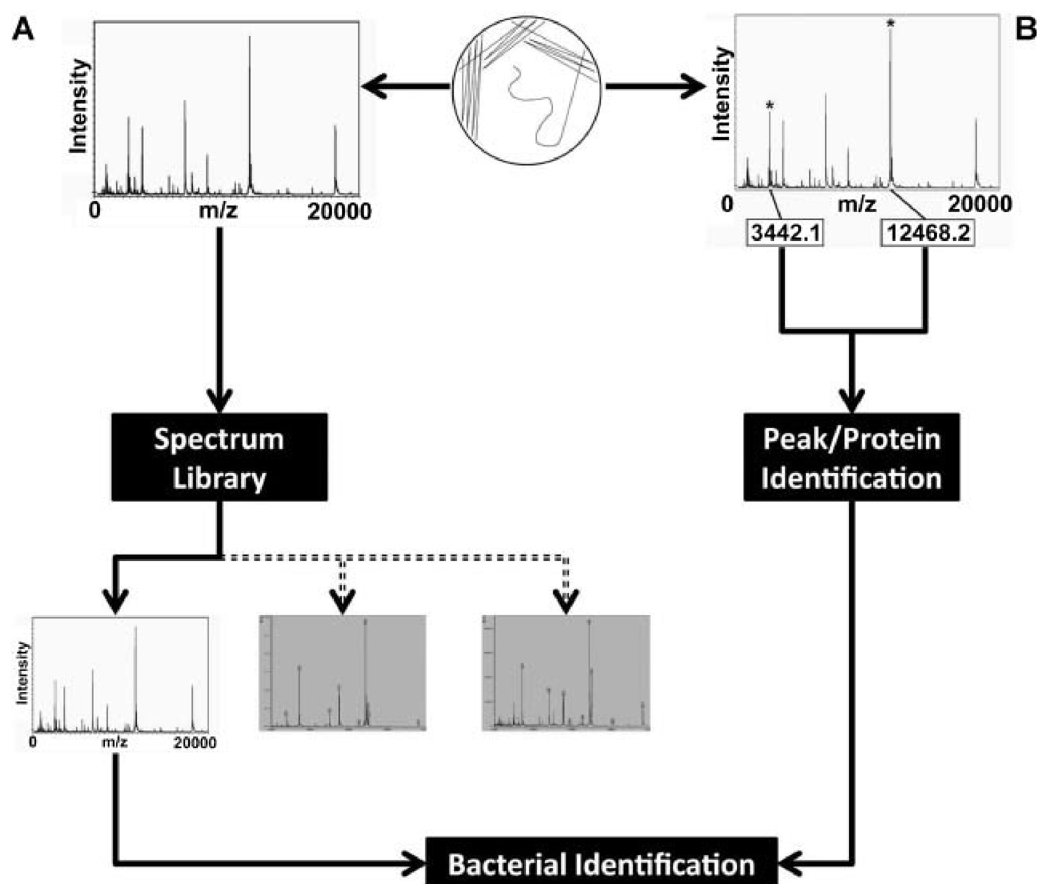

FIGURE 1. Overview of library-based (**A**) and bioinformatics-enabled (**B**) approaches to profiling bacteria using MALDI-MS.

2010), it remains possible that lipids, carbohydrates, and other biological molecules are represented in these spectra. Spectra are often acquired via automation (i.e., instrument operation software, not a human operator, acquires data).

Once spectra are collected, data are analyzed to obtain information that might be useful to characterize/identify the microorganism in question. Spectra of unknown bacteria are compared to libraries of spectra of known bacteria. Holland et al. (1996) were one of the first groups to articulate a need for robust and reliable software algorithms to compare spectra. Currently, commercially available and open source software tools are both available to facilitate comparison of spectra and quantification of similarity between spectra of different bacteria. Meaningful data analysis, extraction, and reduction have been shown repeatedly to be critical to successfully profile bacteria with MALDI TOF MS. Approaches to data analysis are discussed in greater detail later in this review.

2. Bioinformatics-Enabled Approaches

The library-based approach has been used in the majority of studies with MALDI TOF MS to profile bacteria; however, the rapidly increasing number of bacteria with fully sequenced genomes as well as concerns regarding the reproducibility of spectra used in the library-based approach have led many investigators to employ a bioinformatics-enabled approach to bacterial profiling. This approach involves identification of peaks in MALDI profiles of bacteria as particular proteins from publicly available databases with genome sequence data (Fig. 1B). In addition, several bioinformatics-enabled approaches have identified proteins via MS-MS, strategies pioneered by Mortz (1996). Approaches to bacterial profiling with MALDI TOF MS that involve protein identification were later described (Dai et al., 1999; Demirev et al., 1999). Fenselau et al. (2007) have provided an excellent and concise review of the bioinformatics-enabled approach to MS profiling of microorganisms.

The greatest advantage of bioinformatics-enabled approaches is that they do not require construction of libraries of spectra. In addition, conditions do not need to be as rigorously standardized across laboratories as are the case with library-based approaches in which minor variations in protocols can have profound effects on efficacy (Fenselau et al., 2007). Instrumental requirements, though, are often greater with the bioinformatics-enabled approaches than with the library-based approach. Protein and peptide sequence data often used in bioinformatics-enabled approaches to bacterial profiling are most readily obtained with more sophisticated and costly instruments that perform MS-MS (e.g., TOF-TOF) analysis. In addition, the utility of this approach tends to be limited to bacteria

TABLE 1. Efforts to perform bacterial strain categorization with MALDI TOF MS

Bacterium(a)	Approach	Cell preparation	Matrix	Software[1]	Instrument	Reference
cyanobacteria	L	E	CHCA	ns	Voyager Elite; PerSeptive Biosystems	Erhard et al., 1997
Haemophilus	L	I/ E	SA	ns	Voyager-DE; PerSeptive Biosystems	Haag et al., 1998
MRSA/MSSA	L	E	CMBT	ns	Kompact MALDI 2; Kratos Analytical	Edwards-Jones et al., 2000
Salmonella enterica	L	I	CHCA	ns	Voyager DE STR 4071; Perseptive Biosystems	Leuschner, Beresford-Jones & Robinson, 2003
Francisella tularensis	L	E	SA	Bionumerics; Applied Maths	PBS II; Ciphergen Biosystems	Lundquist et al., 2005
MRSA	L	I	CMBT	in-house	Kratos Kompact MALDI 2; Shimadzu Biotech	Jackson et al., 2005
Streptococcus	L	I	CHCA	MATLAB; Math Works	Biflex III; Bruker Daltonics	Rupf et al., 2005
Neisseria gonorrhoeae	L	E	SA	Neuroshell; Ward Systems Group	PBS II Protein Chip Array Reader; Ciphergen Biosystems	Schmid et al., 2005
Moraxella catarrhalis	B	I/E	CHCA	MASCOT	Ultraflex; Bruker Saxonia Analytik	Schaller et al., 2006
Arthrobacter	L	I	SA, CHCA, DHB	Bionumerics; Applied Maths	Reflex III; Bruker Daltonics	Vargha et al., 2006
Escherichia coli	L	I	SA	Diversity Database; Bio-Rad	Reflex IV; Bruker Daltonics	Siegrist et al., 2007
Escherichia coli	B	E	SA	ns	Voyager-DE STR; Applied Biosystems	Camara & Hays, 2007
Enterococcus	L	E	SA	GelComparII; Applied Maths	Reflex IV; Bruker Daltonics	Giebel, Fredenberg & Sandrin, 2008
Listeria	L	E	CHCA	BioTyper, Bruker Daltonics	Microflex LT; Bruker Daltonics	Barbuddhe et al., 2008
Clostridia	L	E	CHCA	BioTyper; Bruker Daltonics	Microflex LT; Bruker Daltonics	Grosse-Herrenthey et al., 2008
Lactobacillus	L	E	DHB	MS-Screener database; mpiib-berlin.mpg	Ultraflex II TOF-TOF; Bruker Daltonics	Schmidt et al., 2009
Rhodococcus erythropolis	L	E	SA	Bionumerics; Applied Maths	Axima CFR; Shimadzu/Kratos	Teramoto et al., 2009
Yersinia pestis	L	I	CHCA	BioTyper; Bruker Daltonics	Autoflex II; Bruker Daltonics	Ayyadurai et al., 2010
Staphyloccus	L	E	CHCA	BioTyper; Bruker Daltonics	Ultraflex II TOF-TOF; Bruker Daltonics	Dubois et al., 2010
MRSA/MSSA	L	E	CHCA	BioTyper; Bruker Daltonics	Microflex LT; Bruker Daltonics	Szabados et al., 2010
Escherichia coli	L	E	CHCA	BioTyper; Bruker Daltonics	Ultraflex I; Bruker Daltonics	Karger et al., 2010
MRSA	L	E	CHCA	BioTyper; Bruker Daltonics	Microflex LT; Bruker Daltonics	Wolters et al., 2011
Frankia	L	E	SA	SARAMIS; bioMérieux	Axima Confidence; Shimadzu-Biotech	Hahn et al., 2011
MRSA	L	E	SA	ns	PBS II Protein Chip Array Reader; Ciphergen Biosystems	Shah et al., 2011
Yersinia enterocolitica	L, B	I	SA	ns	Ultraflex II TOF-TOF; Bruker Daltonics	Kraushaar et al., 2011
Yersinia enterocolitica	L	I	SA	SARAMIS; bioMérieux	Axima Confidence; Shimadzu-Biotech	Stephan et al., 2011

B, bioinformatics-enabled; CHCA, α-cyano-4-hydroxycinnamic acid; CMBT, 5-choloro-2-mercaptobenzothiazole; DHB, 2-hydroxy-5-methoxy benzoic acid; E, extract; I, intact; L, library-based; MRSA, methicillin-resistant *Staphyloccus aureus*; MSSA, methicillin-sensitive *Staphyloccus aureus*; ns, not specified; SA, 3,5-dimethoxy-4-hydroxycinnamic acid (sinapininc acid).

[a]Not an exhaustive list; only unique and/or software packages critically important for strain-level characterization are listed.

TABLE 2. Efforts to perform bacterial strain differentiation with MALDI TOF MS

Bacterium(a)	Approach	Cell preparation	Matrix	Software[1]	Instrument	Reference
Gram (-) Gram (+) *enterobacteriai*	L	I	CHCA	ns	Kompact MALDI III; Kratos Analytical	Claydon et al., 1996
various pathogenic and non-pathogenic	L	E	CHCA, SA	ns	Vestec 2000; Vestec Instruments	Krishnamurthy, Rajamani & Ross, 1996
cyanobacteria	L	I	CHCA	ns	Voyager Elite; PerSeptive Biosystems	Erhard, von Dohren & Jungblut, 1997
Gram (+) Gram (-)	L	I	HABA	ns	ns; Fison Instruments	Welham et al., 1998
Bacillus subtilis	L	I, E	CHCA	ns	Reflex; Bruker Daltonics	Leenders et al., 1999
Enterobacteriaceae	L	I	CHCA	ns	in house	Lynn et al., 1999
Helicobacter pylori	L	E	CHCA, SA, FA	ns	Reflex; Bruker-Franzen	Nilsson, 1999
MRSA, MSSA	L	I	CBMT	ns	Kompact MALDI 2; Kratos Analytical	Edwards-Jones et al., 2000
Bacillus	L	I, E	SA	ns	Kompact MALDI 4; Kratos Analytical	Ryzhov, Hathout & Fenselau, 2001
Bacillus	L	E	CHCA, SA	ns	TofSpec 2E; Micromass Ltd.	Elhanany et al., 2001
MRSA/MSSA	L	E	CHCA, SA, CMBT, MCA	ns	Bruker Reflex III; Bruker Saxonia Analytic	Bernardo et al., 2002
MRSA	L	I	CMBT	ns	Kompact MALDI 2; KratosAnalytica	Walker et al., 2002
Bacillus	L	E	FA	ns	Reflex II; Bruker Daltonics	Dickinson et al., 2004
Bacillus	L	I, E	CHCA, SA, DHB, THAP	ns	TofSpec 2 E; Micromass Ltd.	Horneffer et al., 2004
Acinetobacter, Escherichia coli, Salmonella	L	E	CHCA, SA, CMBT, FA	ns	Tofspec 2E, Micromass Ltd.	Ruelle et al., 2004
Campylobacter	B	E	FA	GPM; Global Proteome Machine	ReflexII; Bruker Daltonics	Fagerquist et al., 2005
Campylobacter	L	E	FA	ns	Reflex II; Bruker Daltonics	Mandrell et al., 2005
Escherichia coli	B	I	CHCA, SA, FA	Masslynx; Waters Corp.	MALDI LR; Micromass Ltd.	Ochoa & Harrington, 2005
Aeromonas	L	I	SA	ns	BiFlex III; Bruker Daltonics	Donohue et al., 2006
Campylobacter	B	E	FA	in-house	Reflex II; Bruker Daltonics	Fagerquist et al., 2006
Mycobacterium	L	E	CHCA	ns	PBS IIC; Ciphergen Biosystems	Hettick et al., 2006
MRSA	L	I	CMBT	MicrobeLynx, Micromass Ltd.	ns; Micromass Ltd.	Majcherczyk et al., 2006

with sequenced genomes; however, the ever-increasing amount of publicly available bacterial genome data should facilitate greater use of bioinformatics-based approaches in the future.

Currently, there is no clear consensus with regard to whether bioinformatics-based approaches perform better than library-based approaches. Library-based approaches have been employed more commonly than bioinformatics-based approaches (Tables 1–3), but this will likely change in the future as: (1) the cost of the requisite hardware to perform bioinformatics-based approaches decreases and (2) publicly available genome sequence data increases.

TABLE 2. (*Continued*)

Lactobacillus plantarum	B	E	SA	ns	n/s	Sun et al., 2006
Bacillus anthracis, Bacillus cereus	B	I,E	CHCA	Data Explorer; Applied Biosystems	Ultraflex I, Bruker Daltonics; 4700 Proteomics Analyzer TOF-TOF; Applied Biosystems	Castanha et al., 2007
Coxiella burnetii	L, B	I	CHCA, SA, CMBT	MATLAB; Math Works Inc.	4700 Proteomics Analyzer TOF-TOF; Applied Biosystems	Pierce et al., 2007
Bacillus	L	I	DHB	ns	Omniflex; Bruker Daltonics	Price et al., 2007
Francisella tularensis	L	E	SA	CIPHERGENEX PRESS, Ciphergen Biosystems	4000 Enterprise Edition; Ciphergen Biosystems	Seibold et al., 2007
Lactobacillus bulgaricus, Streptococcus thermophilus	B	E	SA	ns	Axima CFR; Shimadzu/Kratos	Teramoto et al., 2007a
Pseudomonas putida	B	E	SA	BioNumerics; Applied Maths	Axima CFR; Shimadzu/Kratos	Teramoto et al., 2007b
Escherichia coli	L	I	SA	MATLAB; Math Works	MALDI LR; Micromass Ltd.	Chen, Lu & Harrington, 2008
Escherichia coli, Shigella	L	E	SA	ns	Ultraflex II; Bruker Daltonics	Everley et al., 2008
Streptococcus pyogenes	L	I	CHCA, SA, DHB	ns	AB 4700 Proteomics Analyzer TOF-TOF; Applied Maths	Moura et al., 2008
Streptococcus	L	I	SA	ns	AB 4700 Proteomics Analyzer TOF-TOF; Applied Maths	Williamson et al., 2008
Escherichia coli	B	E	CHCA, SA	in-house	4800 TOF-TOF; Applied Biosystems	Fagerquist et al., 2010
Pseudomonas	B	I	SA	ns	Axima Performance; Shimadzu/Kratos	Hotta et al., 2010
Enterobacteriaceae	L	E	SA	ns	Ultraflex II; Bruker Daltonics	Mott et al., 2010
Lactococcus lactis	B	E	SA	ns	Voyager DE PRO; Perseptive Biosystems	Tanigawa, Kawabata & Watanabe, 2010
Lactic acid bacteria	L	E	CHCA	BioNumerics; Applied Maths	Microflex LT; Bruker Daltonics	Albesharat et al., 2011
Legionella	L	E	CHCA	BioTyper, Bruker Daltonics	Autoflex II; Bruker Daltonics	Fujinami et al., 2011
variety of environmental bacteria	L	E	CHCA	BioNumerics; Applied Maths	4800 Plus TOF-TOF; AB Sciex	Ghyselinck et al., 2011
Bifidobacterium longum	B	E	SA	Bionumerics; Applied Maths	Axima CFR; Shimadzu/Kratos	Sato et al., 2011
Propionic Acid Bacteria	L	I	CHCA, SA, DHB	BioNumerics; Applied Maths	Autoflex II; Bruker Daltonics	Vorob'eva et al., 2011

B, bioinformatics-enabled; CHCA, α-cyano-4-hydroxycinnamic acid; CMBT, 5-choloro-2-mercaptobenzothiazole; DHB, 2-hydroxy-5-methoxy benzoic acid; E, extract; FA, ferulic acid; HABA, 2,4-hydroxyphenylazobenzoic acid; I, intact; L, library-based; MRSA, methicillin-resistant *Staphyloccus aureus*; MSSA, methicillin-sensitive *Staphyloccus aureus*; n/s, not specified; SA, 3,5-dimethoxy-4-hydroxycinnamic acid (sinapininc acid); THAP, 2,4,6-trihydroxyacetophenon.

[a]Not an exhaustive list; only unique and/or software packages critically important for strain-level characterization are listed.

TABLE 3. Efforts to perform bacterial strain identification with MALDI TOF MS

Bacterium(a)	Approach	Cell preparation	Matrix	Software[1]	Instrument	Reference
Escherichia coli K-12	L	E	CHCA	in-house	in-house	Arnold & Reilly, 1998
Various Gram (+) and Gram(-)	L	I	CHCA, CMBT	MUSE™; Manchester Metropolitan University	Kompact MALDI II; Kratos Analytical	Bright et al., 2002
Bordetella, as-yet-uncultured	B	E	3-HPA	SEQUENOM; Sequenom	Biflex DE; Bruker Daltonics	von Wintzingerode et al., 2002
Escherichia coli	B	E	3-HPA	Discovery-RT; Sequenom	MassARRAY; Bruker-Sequenom	Honisch, et al., 2004
Micrococcaceae (CoNS)	L	I	DHB	BGP database	Autoflex; Bruker Daltonics	Carbonnelle et al., 2007
Neisseria meningitidis	B	E	3-HPA	SEQUENOM; Sequenom	ns	Honisch,et al., 2007
Stenotrophomonas maltophilia	L	E	3-HPA & DAC	Data Explorer; Applied Biosystems	Voyager DE-STR; Applied Biosystems	Jackson et al., 2007
Salmonellae	B	I	SA, CHCA, DHB	SARAMIS; bioMérieux	Ultraflex II TOF-TOF; Bruker Daltonics	Dieckmann, et al., 2008
Enterobacteriaceae *Erwinia*	L	E	CHCA	BioTyper; Bruker Daltonics	Ultraflex I; Bruker Daltonics	Sauer et al., 2008
Streptococcus agalactiae	L	E	CHCA	BioTyper; Bruker Daltonique	Ultraflex III TOF-TOF; Bruker Daltonics	Lartigue et al., 2009
MRSA	B	E	3-HPA	MassARRAY Typer; Sequenom	MassArray Compact Analyzer; Sequenom	Syrmis, et al., 2011

B, bioinformatics-enabled; CHCA, α-cyano-4-hydroxycinnamic acid; CMBT, 5-choloro-2-mercaptobenzothiazole; CoNS, coagulase-negative staphylococci; DHB, 2-hydroxy-5-methoxy benzoic acid; E, extract; I, intact; L, library-based; MRSA, methicillin-resistant *Staphyloccus aureus*; ns, not specified; SA, 3,5-dimethoxy-4-hydroxycinnamic acid (sinapininc acid); 3-HPA & DAC, hydroxypicolinic acid and diammonium citrate.

[a]Not an exhaustive list; only unique and/or software packages critically important for strain-level characterization are listed.

B. Successes at the Genus and Species Levels

Library- and bioinformatics-enabled approaches to bacterial profiling at the genus and species levels have both yielded results that are comparable to or superior to those obtained from more conventional approaches. Such successes can be found across diverse areas of research and disciplines that include clinical microbiology, biodefense, food safety, and environmental health.

Applications of MALDI TOF MS profiling of bacteria in clinical microbiology at the species and genus levels are manifold. For example, Mellmann et al. (2008) constructed a library of 248 culture collection strains of non-fermenting bacteria, several of which were opportunistic pathogens. Eighty blind-coded samples were used to test the effectiveness of this approach. Ninety-five percent of these samples were identified to the correct genus level, whereas 83% were identified to the correct species level. Results were in general agreement with 16S rDNA sequence data. In fact, characterization of some bacteria, particularly members of the *Burkholderia cepacia* complex, was more accurate with MALDI TOF MS than 16S rDNA sequence data. The method was found to be particularly reproducible, even across different laboratories (Mellmann et al., 2009). Rapid identification of *S. aureus* among a collection of 602 strains within this genus has been reported (Szabados et al., 2010). Most recently, Mukhopadhya et al. (2011) used a library-based approach to characterize strains of a potential bacterial trigger of inflammatory bowel disease (IBD), *Sutterella wadsworthensis*. Applications of library-based approaches have not been limited to bacteria that affect humans. MALDI TOF MS profiling at the species level has facilitated studies in wildlife biology. For example,

Rubbenstroth et al. (2011) recently used MALDI TOF MS to rapidly distinguish between two species within a genus of a pigeon pathogen, *Riemerella*.

MALDI TOF MS profiling of bacteria at the genus and species levels has shown particular promise in biodefense applications. Several investigators have successfully identified biomarkers of spores of the causal agent of anthrax, *Bacillus anthracis* (an excellent review is provided by Stump et al., 2005). For example, Elhanany et al. (2001) reported biomarkers specific to *B. anthracis*, while English et al. (2003) subsequently used a small, custom, MALDI TOF MS unit for rapid detection of *B. anthracis*. An excellent review of additional applications of bacterial profiling via MALDI TOF MS in biodefense is available (Demirev & Fenselau, 2008b). Several studies have reported strain-level biomarkers that are described in more detail later in this review. With regard to food safety, a library-based approach was used recently to identify several bacterial contaminants in seafood products (Böhme et al., 2010, 2011).

In the area of environmental health, Fox et al. (2011) recently employed a bioinformatics-enabled approach to identify to the species level coagulase negative staphylococci from indoor air. Library-based approaches have been used to identify *Legionella*, the bacterial genus that contains the causal agent of Legionnaire's disease, in water from cooling towers (Pennanec et al., 2010). Similarly, a library-based approach has been used to elucidate environmental sources of members of the genus *Enterococcus* (a bacterial indicator of water quality) in recreational waters (Giebel, Fredenberg, & Sandrin, 2008). Species-level bacterial profiling has also facilitated research in microbial ecology. Munoz et al. (2011) recently used a library-based approach to characterize 374 moderately halophilic bacteria that dwell in hypersaline sediments.

C. Strain-Level Resolution: Needs and Challenges

Although characterization at the genus and species levels is adequate for many applications that include those described above, discrimination at the strain level is required for many applications. Epidemiologic investigations require rapid identification of a single strain within a single species to determine the origin and spread of an outbreak (Tenover, Arbeit, & Goering, 1997; Murray, 2010). Microbial forensics requires strain-level resolution to track origins of single strains of microorganisms. For example, determination of the environmental source of microbial contaminants in drinking and recreational waters (i.e., source-tracking) requires characterization of many strains of a single species of a microbial water quality indicator, such as *Escherichia coli*. Similarly, identification of individual strains is important in source-tracking in food microbiology to monitor and characterize transmission of flavor-forming bacteria and bacteria responsible for food spoilage.

Although success in profiling bacteria with MALDI TOF MS at the species and genus levels has been obtained with a wide variety of bacteria, success at the strain level has proved more elusive. Several studies have reported an inability to characterize bacteria below the species level (Rezzonico et al., 2010; Gaia, Casati, & Tonolla, 2011). The difficulty lies primarily in the fact that members of a single species tend to yield remarkably similar MALDI TOF MS profiles. As taxa become more specific (i.e., moving from species to strain level), the discriminatory power and resolution of the method must increase. Although many studies have reported strain-specific peaks (as described in more detail below), such biomarker peaks represent a very small portion of the MS profile. Poor and/or not-quantified profile reproducibility can hinder identification of reliable peaks as strain-specific biomarkers. Indeed, MS profile quality, data richness, reproducibility, and mass accuracy must typically exceed levels necessary for species-level profiling (Dieckmann et al., 2008). Further, it appears that the limits of the taxonomic resolution of MALDI TOF MS profiling might be determined in large part by the nature of the particular bacterium profiled (Ghyselinck et al., 2011). Some bacteria are remarkably different genetically at the subspecies levels, whereas others are nearly indistinguishable (Tanigawa, Kawabata, & Watanabe, 2010).

II. APPROACHES AND APPLICATIONS

The literature contains three overarching objectives of bacterial profiling at the strain level (Fig. 2): (1) strain categorization (Fig. 2A; Table 1), (2) strain differentiation (Fig. 2B; Table 2), and (3) strain identification (Fig. 2C; Table 3). Strain categorization groups together similar strains that share a particular trait, environmental origin, or subspecies taxon (e.g., biotypes, serovars). Discrimination of single strains is not the objective of studies that have performed strain categorization. In contrast, single strains are distinguished from one another by the presence and/or absence of one or more discriminating peaks in studies that perform strain differentiation. In studies that perform strain identification, several known strains are used to create a reference library that affords identification of unidentified strains or blind-coded strains from the reference library. Software and/or advanced statistical tools (e.g., artificial neural networks, random forests, etc.) are used to quantitatively compare profiles of unknown strains to those in the reference library. Generally, the requisite discriminatory power is least with strain categorization, intermediate with strain differentiation, and greatest with strain identification. Some investigators have pursued more than one objective in a single study, such as strain categorization and strain differentiation.

A. Library-Based Approaches

Library-based approaches have been employed more frequently than bioinformatics-enabled approaches to obtain strain-level resolution with MALDI TOF MS profiling (Tables 1–3). Several diverse and successful strategies have used library-based approaches to facilitate strain categorization, differentiation, and identification. These strategies are listed in Tables 1–3 and highlighted below.

1. *Strain Categorization*

There is clear need in medical and clinical microbiology for rapid methods to categorize bacterial strains with regard to their origin, antibiotic resistance, and/or pathogenicity. Haag et al. (1998) were one of the first groups to use MALDI TOF MS to categorize strains of medically relevant bacteria (Table 1). The authors reported differences in spectra obtained from *Haemophilus ducreyi* that allowed categorization of the

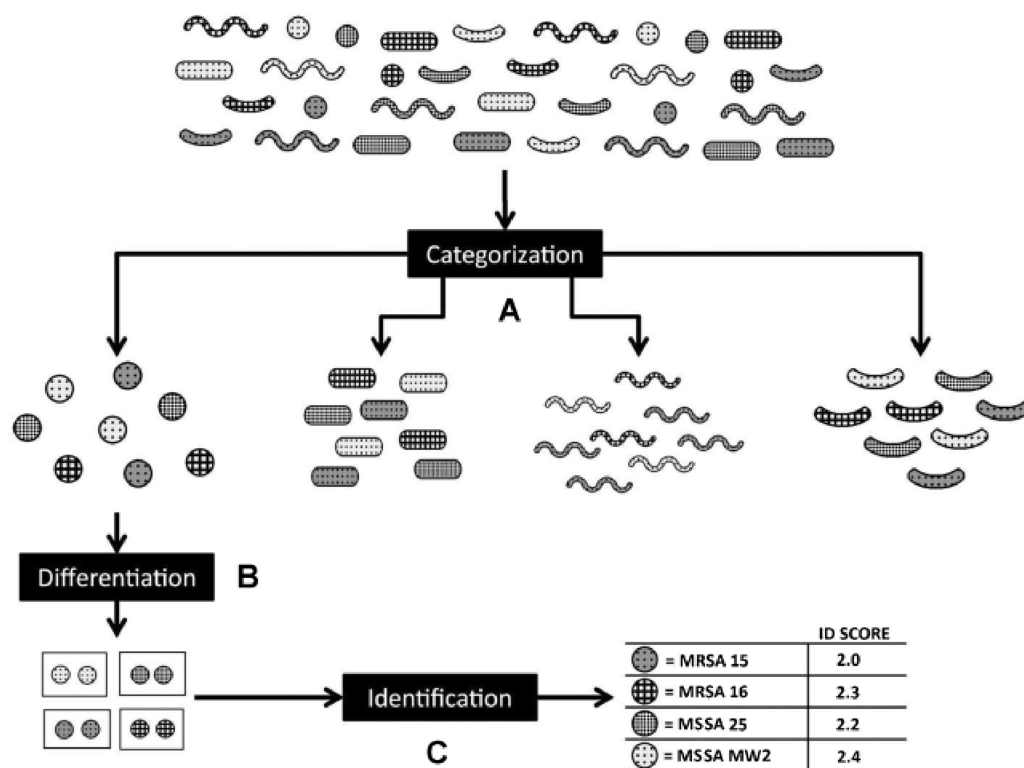

FIGURE 2. Three common objectives of strain-level characterization of bacteria using MALDI-MS: (**A**) strain categorization, (**B**) strain differentiation, and (**C**) strain identification. In strain categorization (A), strains are grouped together with similar strains (e.g., with regard to biotype, serovar, etc.), and single strain resolution is not necessarily obtained. In strain differentiation (B), strains are each distinguished from one another, often by the presence and/or absence of one or more discriminating peaks. In strain identification (C), several strains are used to create a reference library that is designed to afford identification of unidentified strains or blind-coded strains from the reference library. Typically, software and/or statistical tools are used to compare profiles of unknown strains to those in the reference library. Different cell morphologies shown in the figure are used only for illustrative purposes. Strain-level profiling typically provides characterization of strains on the basis of phenotypic and/or genotypic characteristics not related to cell morphology (e.g., antibiotic resistance).

strains with respect to the date of infection. Leuschner, Beresford-Jones, and Robinson (2003) later categorized *Salmonella enterica* subsp. *enterica* serovars (common foodborne-pathogens) through software-enabled comparisons of similarities and differences in peak-rich MALDI TOF MS profiles (up to 500 peaks per profile) of strains of interest. Similarly, three serovars of shiga-toxin producing *E. coli* were characterized by Karger et al. (2010). Strains of yet another important foodborne-pathogen, *Yersinia enterocolitica*, were recently categorized as pathogenic or non-pathogenic (Stephan et al., 2011) with MALDI TOF MS. Similarly, strains of *Francisella tularensis*, the highly infective causal agent of tularemia and a potential bioweapon, have been classified with respect to their membership in one of four subspecies (Lundquist et al., 2005). Strains of another potential bioweapon, the causal agent of plague, *Yersinia pestis*, have been categorized into three biotypes (Ayyadurai et al., 2010). Successful categorization at the subpopulation level has been reported with 18 clinical strains of *Moraxella catarrhalis* (Schaller et al., 2006).

The genus *Staphylococcus* contains members of extraordinary medical importance. MRSA represents a threat to patient welfare throughout the world. MALDI TOF MS profiling has shown promise as a powerful tool to categorize strains of this bacterium. Jackson et al. (2005) developed an optimized protocol that allowed categorization of several MRSA strains according to their relevant PFGE reference type. With a larger (152) and more diverse collection of *Staphylococcus* strains, Dubois et al. (2010) noted that profiles of strains of *S. epidermidis* grouped together with respect to origin (clinical or environmental). With regard to MRSA, Wolters et al. (2011) exploited differences between strains of this antibiotic-resistant pathogen to categorize 60 strains with respect to membership in clonal complexes. Categorization of *S. aureus* as methicillin resistant or sensitive was reported first by Edwards-Jones et al. (2000). More recently, Shah et al. (2011) employed Artificial Neural Networks to categorize successfully 97 of 99 *S. aureus* strains as methicillin resistant or sensitive.

Strain categorization via MALDI TOF MS profiling has not been limited to medical and clinical microbiological applications. Several successes with this approach to categorize bacteria important in environmental microbiology and biotechnology have been reported. With regard to environmental microbiology, Siegrist et al. (2007) employed MALDI TOF MS

profiling to categorize environmental strains of the water quality indicator *E. coli* with regard to its host (bovine, canine, avian, or human). In the realm of terrestrial environmental microbiology, 37 strains of the nitrogen-fixing actinomycete, *Frankia*, were recently categorized with respect to their appropriate infection groups (Hahn et al., 2011). With regard to biotechnological applications, Teramoto et al. (2009) categorized 21 strains of antibiotic-producing *Rhodococcus erythropolis* among three groups.

2. *Strain Differentiation*

Although strain categorization is sufficient for many applications (e.g., determination of whether a particular strain is pathogenic or non-pathogenic), many scenarios require greater resolution and differentiation of individual strains from others. Interestingly, some of the earliest applications of MALDI TOF MS to bacterial profiling facilitated differentiation of individual bacteria at the strain level (Claydon et al., 1996; Krishnamurthy, Rajamani, & Ross, 1996). Although these early studies dealt with a limited number of strains, several subsequent studies dealt with more comprehensive and extensive collections. Examples of successful applications of MALDI TOF MS enabled strain differentiation to clinical and medical microbiology, biodefense, and environmental microbiology and biotechnology are summarized below and in Table 2. In many of these studies (Ruelle et al., 2004; Everley et al., 2008; Jabbour, 2011), one or more peaks are identified as strain-specific biomarkers. In others (Schmid et al., 2005), more sophisticated analyses are performed that do not rely upon a limited number of biomarker peaks.

The promise of MALDI TOF MS profiling of bacteria as a rapid, cost-effective alternative to traditional, time-consuming approaches to differentiate strains of medically and clinically relevant bacteria has stimulated considerable interest in this area. Nilsson (1999) was first to report strain-differentiating biomarkers for the causal agent of stomach ulcers, *Helicobacter pylori*. Similarly, biomarkers were reported quite early in the advent of MALDI TOF MS profiling of bacteria; Lynn et al. (1999) described unique biomarkers that differentiated two strains of *E. coli*. Some strains of the foodborne pathogen-containing genus, *Campylobacter*, yielded strain-differentiating peaks (Mandrell et al., 2005). Strain-differentiating MALDI TOF MS profiles have been reported for MRSA by several groups (Edwards-Jones et al., 2000; Bernardo et al., 2002; Walker et al., 2002; Majcherczyk et al., 2006).

More rigorous analysis of MALDI profiles has yielded strain-level differentiation of diverse bacteria of clinical relevance. Hettick et al. (2006) used linear discriminant analysis and random forest analysis (described in more detail later in this review) to differentiate 16 strains that represent four species of *Mycobacteria*. Interestingly, unique biomarkers differentiated at the species level, but not at the strain level. Instead, consideration of peaks shared among strains was required for strain differentiation. Random forest analysis and identification of unique, strain-specific biomarkers have also been used to differentiate strains of *Streptococcus pyogenes* (Moura et al., 2008) and *S. pneumoniae* (Williamson et al., 2008).

The rapidity of MALDI TOF MS profiling and successes with other bacteria prompted several investigations into strain-differentiating applications of MALDI TOF MS profiling to bacteria that represent possible bioterror agents. Strain-level differentiation of the causal agent of anthrax, *Bacillus anthracis*, has been reported by Elhanany et al. (2001) and Krishnamurthy et al. (2007). Similar successes have been reported with other possible agents of bioterror including the causal agent of Brucellosis, *Brucella melitensis*, the causal agent of bubonic plague, *Y. pestis* (Krishnamurthy, Rajamani, & Ross, 1996), the causal agent of tularemia, *Francisella tularensis* (Seibold et al., 2007), and the causal agent of Q fever, *Coxiella burnetii* (Pierce et al., 2007).

MALDI TOF MS strain differentiation has also benefited the field of environmental microbiology. Ruelle et al. (2004) developed and employed a standardized set of MALDI TOF MS parameters to identify *E. coli*, *Salmonella*, and *Acinetobacter* obtained from sewage sludge. Several environmental strains of *E. coli*, *Salmonella*, and *Acinetobacter* shared biomarker peaks with reference strains, but seven environmental strains of *E. coli* exhibited some unique strain-differentiating biomarkers. Later, several species of another waterborne pathogen, *Aeromonas*, were differentiated at the strain level (Donohue et al., 2006). Moving from the hydrosphere to the lithosphere, Ghyselinck et al. (2011) examined the taxonomic resolution of MALDI TOF MS profiling with bacteria indigenous to the rhizosphere of potato plants. Although the authors reported that they were able to differentiate strains with MALDI TOF MS profiling, the taxonomic resolution of the method appeared dependent upon the particular bacterium under investigation. For example, strains of the genera *Stenotrophomonas*, *Bacillus*, and *Rhodococcus* were differentiated more readily with MALDI TOF MS than a conventional approach (rep-PCR). Similarly, strains of *Enterococcus faecium* and *E. faecalis* isolated from fermented food, breast milk, and feces were differentiated at the strain level with MALDI, but not with a more conventional approach (randomly amplified polymorphic DNA, RAPD; Albesharat et al., 2011). The ability of MALDI TOF MS profiling to differentiate at the strain level does not always exceed that of conventional techniques. Ghyselinck et al. (2011) reported that rep-PCR facilitated strain differentiation more readily than MALDI TOF MS when members of the genera *Rhizobium*, *Streptomyces*, *Paenibacillus*, *Arthrobacter*, and *Pseudomonas* were considered.

3. *Strain Identification*

Identification of individual strains of bacteria often requires greater resolution than strain categorization or strain differentiation. In studies to identify individual, unknown (or blind-coded) strains, profiles of unknown strains were compared against a collection of profiles of reference strains. Single peaks (biomarkers) used to differentiate one strain from another or to categorize one strain within a group have often not proven adequate to allow confident identification of a single strain against a library of many known, reference strains. Strain identification often requires analysis of the entire spectrum rather than one or a few biomarker peaks. In addition, strain identification is often facilitated with software that employs advanced statistical analyses.

Interestingly, one of the earliest reports of success with MALDI TOF MS for bacterial profiling at the strain level

reported strain identification (Arnold & Reilly, 1998). Twenty-five strains of *E. coli* were readily identified with a mathematical algorithm that relied upon statistical correlation. Arnold and Reilly's approach was particularly effective and ahead of its time in that it recognized that intense features and peaks could be given too much weight by standard correlation functions. Instead, the authors enhanced the sensitivity of the correlation analysis to minor differences between spectra. A subsequent, larger study identified 212 bacteria that represented 12 genera (Bright et al., 2002). With pattern recognition software that considered the entire spectrum, 79% of samples were identified correctly to the strain level. Carbonnelle et al. (2007) later employed a similar library-based approach for strain-level identification of members of the family *Micrococcaceae* (including members of the genus *Staphylococcus* and *Micrococcus*). The authors gathered spectra from 10 colonies, each from a separate culture. Only peaks conserved among these 10 spectra were used in the library constructed to identify unknowns. Sauer et al. (2008) further recognized the importance to focus on particular portions of spectra to enhance strain identification of members of the genus *Erwinia*, which contains plant pathogenic members. Strain-level identification required implementation of weighted pattern matching, in which peaks deemed important to discriminate strains were given more weight in the analysis. Dieckmann et al. (2008) employed a similar approach in which biomarker peaks were given more weight than non-specific peaks. Strain identification of *Erwinina* (Sauer et al., 2008) and later of *Streptococcus agalactiae* (Lartigue et al., 2009) were facilitated by the commercially available bacterial identification software, BioTyper (Bruker Daltonics, Billerica, MA), discussed in more detail later in this review.

4. Approaches to Increase Profile Data Richness and Quality

Several investigators have attempted to increase the discriminatory power of library-based approaches with increased numbers of biomarker peaks (i.e., peak richness) in MS profiles. Ryzhov, Hathout, and Fenselau (2000) employed sonication and corona plasma discharge (CPD) to maximize the number of possible biomarker peaks obtained from spores of *Bacillus cereus* group spores (Fig. 3). Spores treated with CPD or sonication yielded spectra that contained biomarker peaks that differentiated several strains. Wet-heat treatment increased the number of biomarkers for different strains of *B. subtilis* and *B. cereus* (Horneffer et al., 2004). Enzymatic pre-treatment of cells increased spectrum quality and biomarker abundance. Krishnamurthy, Rajamani, and Ross (1996) used trypsin digestion of cells to augment peak lists and differentiate between strains of the genus *Bacillus*, including strains within the species *anthracis*. Interestingly, Vargha et al. (2006) reported that lysozyme pre-treatment of cells reduced spectrum quality (base peak signal-to-noise ratio, S:N). Conversely, Giebel, Fredenberg, and Sandrin (2008) reported that pre-treatment of *Enterococcus* sp. strains with lysozyme increased base peak S:N and overall reproducibility. Nilsson (1999) used a detergent, *n*-octylglucoside, to increase the number of large protein biomarkers for strains of *H. pylori*. The authors reported detection of strain-differentiating biomarkers observed in a broader mass range (up to 40,000 Da) than that which is commonly employed.

Reasoning that peak quality is more important than peak quantity, several groups have attempted to increase the discriminatory power of library-based approaches by reduction of number of peaks in MS profiles. Schaller et al. (2006)

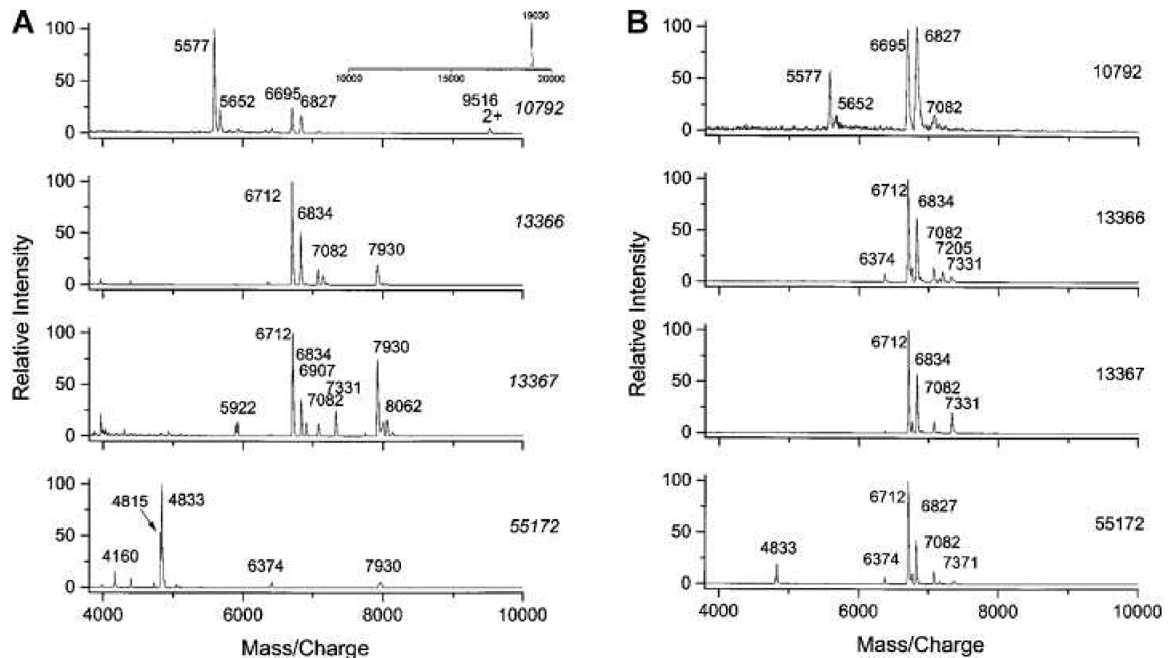

FIGURE 3. Corona plasma discharge (CPD) treatment enhanced biomarker discovery in *Bacillus* spores. CPD-treated spores (**B**) yielded peaks not observed in spectra of untreated spores (**A**). Adapted from Ryzhov and Fenselau (2001).

discriminated among strains of *Moraxella catarrhalis* by first separating outer membrane proteins from cells with two-dimensional gel electrophoresis (2D GE). Profiles of extracted outer membrane proteins alone were sufficient for strain-level categorization. In fact, profiles from extracted outer membrane proteins alone and from intact cells were remarkably similar; that study suggested that the vast majority of peaks that arise from intact cell profiling originate from the outer membrane.

Several groups have reduced peak number with surface-enhanced laser desorption/ionization (SELDI) TOF MS. In this approach (Fig. 4), extracts of bacterial strains of interest are applied to a SELDI array that exhibits unique chromatographic properties. Arrays vary with respect to hydrophobicity, ion-exchange capabilities, and/or metal affinity (Bio-Rad Laboratories, Hercules, CA) and capture only a subset of the bacterial proteome (i.e., that portion of the proteome that has affinity for the surface of the array; Hutchens and Yip, 1993; Tang, Tornatore, & Weinberger, 2004; Wiesner, 2004). Lundquist et al. (2005) later Seibold et al. (2007) reported success with SELDI to discriminate four strains of *Francisella tularensis* (Fig. 5). Strains were discriminated quite effectively, but such discrimination required multiple SELDI arrays. Later work by Schmid and colleagues employed a SELDI-based approach with a variety of bacterial pathogens, including *Neisseria gonorrhoea* (Schmid et al., 2005). Clear strain-level resolution was not obtained, and some overlap between different species was observed. More recently, Shah et al. (2011) characterized 99 *S. aureus* strains with regard to their methicillin resistance with SELDI. Nearly all (98%) of strains were characterized correctly with regard to their methicillin resistance. In addition, the authors reported that this approach appeared capable to detect several strains that transitioned from a state of methicillin susceptibility to one of resistance. Although these results were promising, the authors noted that more widespread use of SELDI-based approaches is hindered by the fact that SELDI arrays are currently compatible with only a single manufacturer's instrument (formerly Ciphergen, now Bio-Rad).

Data analysis techniques that focus on particular portions of MS profiles have also proven useful to resolve bacterial strains, as mentioned earlier. Arnold and Reilly's (1998) modified correlation approach focused on portions of the profiles

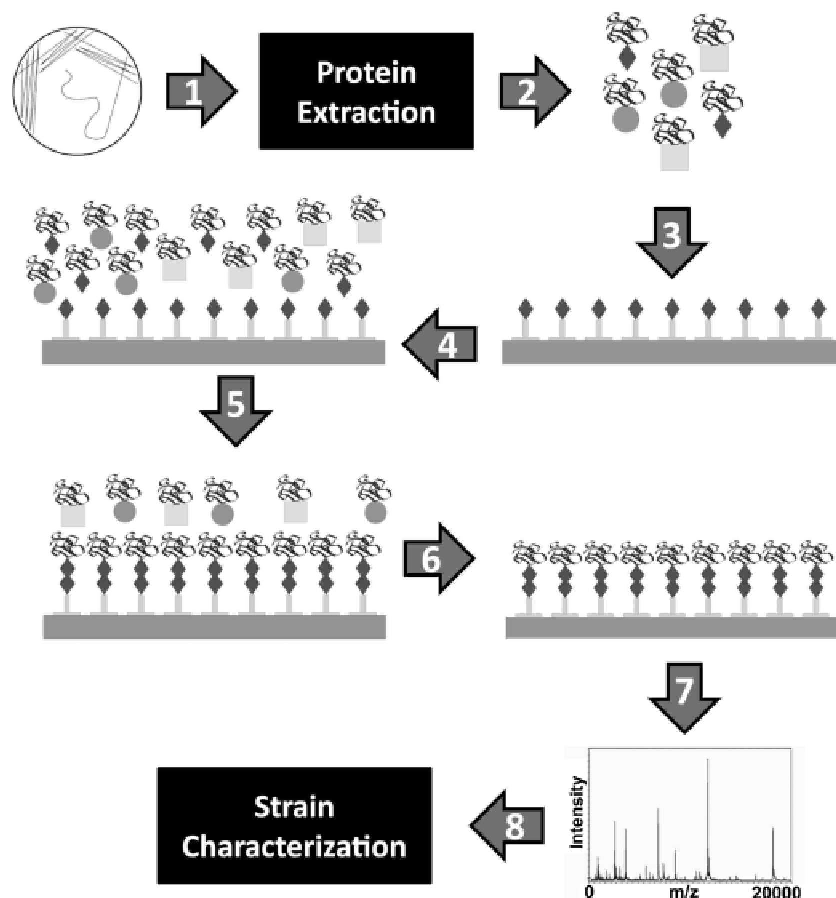

FIGURE 4. Overview of the surface-enhanced laser desorption/ionization (SELDI) approach to microbial fingerprinting using MALDI-MS. Proteins are extracted from bacteria of interest (1). Extracted proteins vary with respect to particular properties such as hydrophobicity or metal affinity, depicted by different shapes (circles, squares, or diamonds) (2). The SELDI target (3) has affinity for a subset of the extracted proteins (4). Proteins lacking affinity for the SELDI target surface do not bind to the SELDI target surface (5), and are washed from the target (6). MALDI-MS is used to profile proteins bound to SELDI target (7). Characterization of proteins on the SELDI target facilitates strain characterization (8).

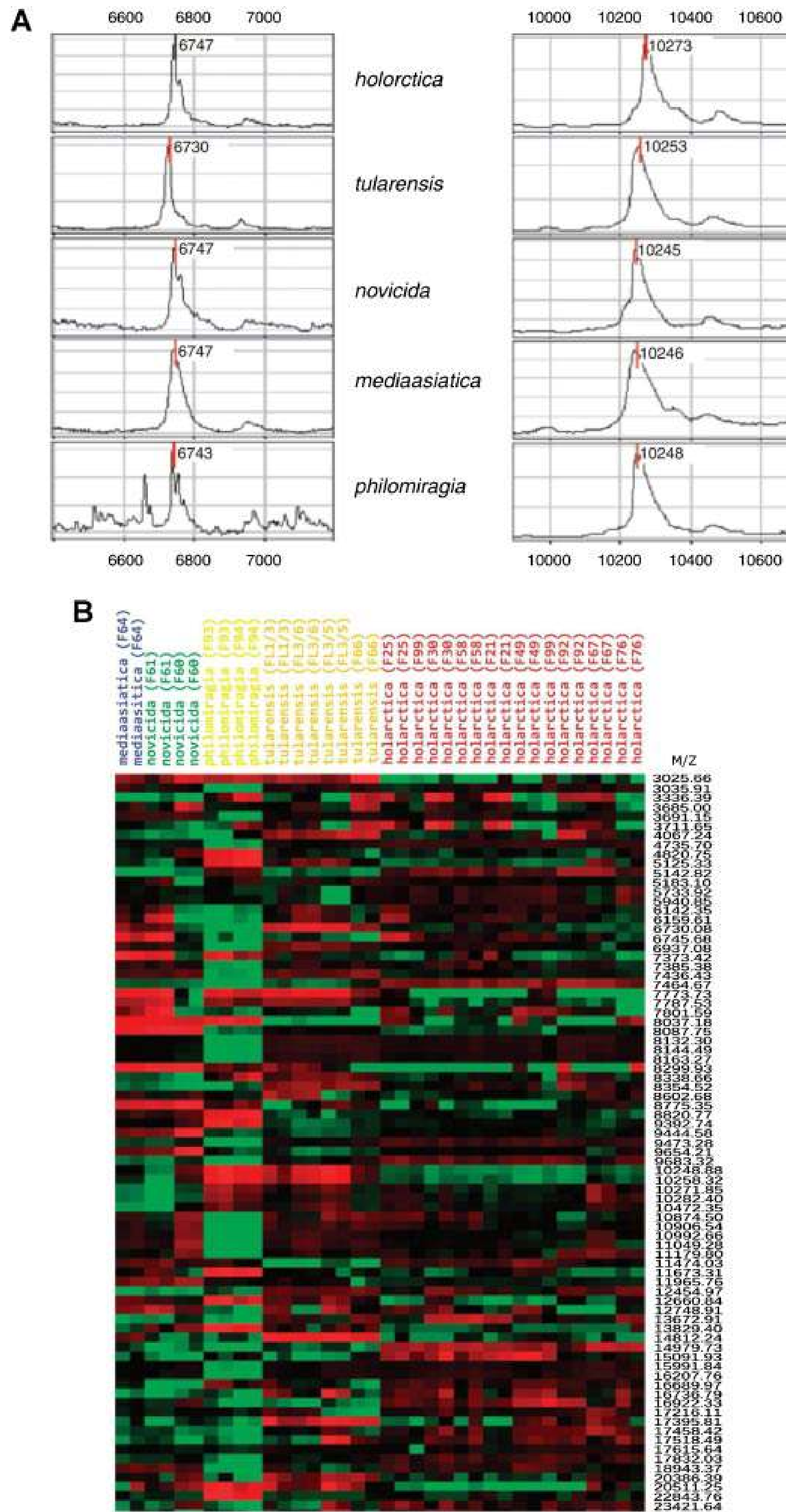

FIGURE 5. Surface-enhanced laser desorption/ionization (SELDI)-facilitated resolution of strains of *Francisella tularensis*. Mass shifts (**A**) and cluster analysis (heat map view) (**B**) reveal strain-level differences. Peak intensity is represented in the heat map using color in which red designates high intensity, black designates medium intensity, and green represents low intensity/absent peak. Adapted from Seibold et al. (2007).

that contained minor differences to ensure that intense peaks did not overly influence their correlation statistic. Carbonnelle et al. (2007) considered only peaks that consistently appeared across profiles of 10 cultures (passages). Sauer et al. (2008) used a weighted-pattern matching approach to focus on portions of MALDI TOF MS profiles that were deemed most useful to discriminate species of *Erwinia*.

B. Bioinformatics-Enabled Approaches

The ever-increasing availability of genome-sequence data for bacteria has inspired many investigators to employ bioinformatics-enabled approaches to profile bacteria at the strain level with MALDI TOF MS. Bioinformatics-enabled approaches use genome sequence data to identify proteins that are useful to characterize bacterial strains. Several strain-resolving, intact protein biomarkers observed in library-based approaches described above have been identified by comparison of their masses to those of proteins predicted in silico from genome sequence data (Fig. 6A). In what have been termed bottom-up approaches (Fig. 6B), enzymatic or chemical digestion is used to digest pre-fractionated proteins into peptides that are analyzed with mass spectrometry (Eng, McCormack, & Yates, 1994). Peptides, as well as the proteins and bacteria from which they originated, can be identified in this approach with tandem mass spectrometry, post-source decay (PSD), in-source decay (laser-induced dissociation), or collisionally induced decay (CID). In top-down applications (Fig. 6C), tandem mass spectrometry (MS-MS) is used to fragment intact proteins into smaller peptides. Strain identification is obtained by comparison of the MS-MS spectrum with in silico-generated spectra contained within a proteome database of a bacterium of interest (Mortz, 1996). Several investigators have employed library-based and bioinformatics-enabled approaches to profile bacterial strains (Dickinson et al., 2004; Mandrell et al., 2005; Schaller et al., 2006; Schmidt et al., 2009). Bioinformatics-enabled approaches to microbial characterization were pioneered by Dai et al. (1999) and Demirev et al. (1999), but strain-level applications were not more widely reported until the mid-2000s (Fagerquist et al., 2005). Successes and strategies that use bioinformatics-enabled approaches are highlighted below and listed in Tables 1–3.

1. Intact Biomarker Identification

Strain-resolving biomarkers have been routinely identified by a comparison of the masses of these intact biomarkers to those predicted in silico. Ochoa and Harrington (2005) identified several strain-specific protein biomarkers observed in pure cultures of *E. coli* O157:H7 and from this bacterium obtained via immunomagnetic separation from meat. Proteins were identified by comparison with masses of predicted proteins contained in the TrEMBL/Swiss-Prot database (Bairoch & Apweiler, 1996). Similarly, Mandrell et al. (2005) identified species identifying biomarker ions (SIBIs) that varied between strains of another foodborne pathogen, *Campylobacter.* The authors confirmed the identity of some SIBIs with a bottom-up approach (peptide mass fingerprinting, PMF). Identification of intact biomarkers supported strain-level resolution of 126 strains of *Salmonella* (Dieckmann et al., 2008). Protein

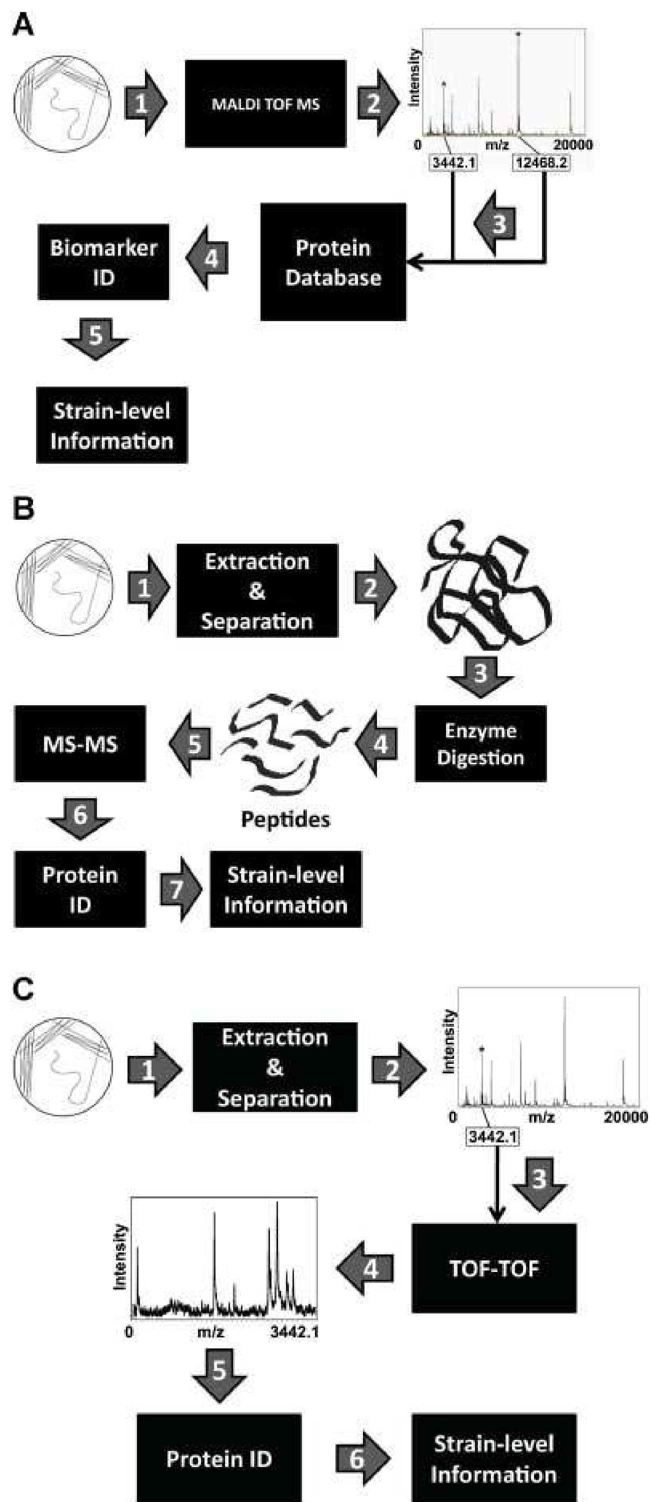

FIGURE 6. Bioinformatics-enabled approaches to strain-level profiling of bacteria using MALDI-MS. Intact biomarker identification (**A**), bottom-up (**B**), and top-down (**C**) approaches are shown.

biomarkers were identified mostly as ribosomal proteins or nucleic acid-binding proteins.

Several bioinformatics-enabled approaches to MS profiling at the strain level have focused on subsets of the bacterial

proteome. Most commonly, ribosomal proteins are used for this purpose. Teramoto et al. (2007a) isolated strain-specific ribosomal proteins in genome-sequenced strains of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, bacteria commonly used as starter cultures for yogurt production. Ribosomal protein purifications were profiled with MALDI and identified by comparison to ribosomal proteins contained in the TrEMBL/Swiss-Prot database. This level of taxonomic resolution was particularly impressive when one considers that genes (e.g., 16S, *gyrB*, and *dnaJ*), commonly used to profile these bacteria genetically are remarkably similar. Teramoto et al. (2007b) later employed a similar approach that relied upon ribosomal proteins to profile strains of *Pseudomonas putida*. Hotta et al. (2010) more recently extended this application to members of the genus *Pseudomonas* without fully sequenced genomes. The authors differentiated strains by creating a database of ribosomal proteins, and focused on a single operon of this genus. Identification of ribosomal proteins with a publicly available database (Rapid Microorganism Identification Database; http://rmidb.org) that accounts for possible post-translational modifications to ribosomal proteins has been used to differentiate among 25 strains of the probiotic bacteria *Lactococcus lactis* (Tanigawa, Kawabata, & Watanabe, 2010).

2. Bottom-Up Approaches

Identification of biomarker proteins to facilitate strain-level profiling is more commonly accomplished with bottom-up approaches than with the intact biomarker approaches described above. Although this approach is relatively labor-intensive, and often requires a pre-fractionation step in addition to a time-intensive enzymatic digestion step, promising results have been reported. It should be noted that many studies not described in detail in this review have used alternatives to MALDI TOF MS, such as ESI MS, to identify strain-characterizing biomarkers (Castanha et al., 2007; Krishnamurthy et al., 2007; Dworzanski et al., 2010; Jabbour et al., 2010).

Dickinson et al. (2004) attempted to identify biomarker peaks in the MALDI TOF MS profiles of *B. subtilis*, but noted that assignment of these proteins to peaks observed in the intact cell profile was challenging. Dickinson and colleagues also noted that prior separation of proteins before bottom-up approaches were employed, was necessary to more confidently relate identified proteins to those observed in intact cell MALDI TOF MS profiles. Fagerquist et al. (2005) separated protein extracts of *Campylobacter* with HPLC and one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D SDS–PAGE) prior to identification with a bottom-up approach. These additional purification steps allowed the investigators to confidently identify a 10 kDa biomarker as a DNA-binding protein. The molecular weight of the protein that varied across strains facilitated strain differentiation. Schaller et al. (2006) also pre-fractionated proteins prior to identification, but used 2D GE instead of the simpler 1D approach. A similar labor-intensive approach to profile *Lactobacillus plantarum* cultures at the strain level, with 2D GE and PMF, has been reported (Sun et al., 2006). Camara and Hays (2007) pre-fractionated proteins with 1D SDS–PAGE and identified with PMF the protein to confirm ampicillin resistance (β-lactamase) in an ampicillin-resistant strain of *E. coli*.

Protein identification has not been performed in all bottom-up approaches to strain-level profiling. With shotgun mass mapping, Schmidt et al. (2009) differentiated strains of *Lactobacillus* with tryptic digestions of cells of reference strains of *Lactobacillus* and strains from dental patients' teeth. The resulting digests were profiled with MALDI TOF MS, and a library of mass spectra was created and used to categorize unknown strains with regard to their relevant subspecies.

3. Top-Down Approaches

The time- and labor-intensive nature of bottom-up approaches to MALDI profiling of bacteria at the strain level has prompted interest in more time-efficient top-down approaches. The instrumentation, however, required to perform top-down approaches must perform tandem MS-MS. Such instrumentation is considerably more costly than many of the simpler MALDI TOF MS instruments more routinely used for bacterial characterization. The more significant cost of the instrumentation seems to have limited more widespread application of top-down approaches to profile bacteria at the strain level. Although Demirev and colleagues pioneered this approach to bacterial profiling several years ago, few strain-level applications with MALDI have been described (Demirev et al., 2005). Top-down approaches might become more common when more economical TOF-TOF units become available.

Fagerquist et al. (2010) employed a MALDI TOF-TOF instrument to identify protein biomarkers that distinguished three strains of *E. coli*—two were pathogenic O157:H7 and one non-pathogenic. Primary sequences of the biomarker in the pathogenic strains were different from those in the non-pathogenic strain. Moreover, the approach facilitated identification of a biomarker present only in non-pathogenic strains. Later, Fagerquist and Sultan (2011) used a similar method to identify two different forms of shiga toxin produced by *E. coli* O157:H7. It seems possible that a similar approach might be used in the future to perform strain-level characterization through broader, rapid identification of toxins and metabolites that are produced in a strain-specific manner.

4. Nucleic Acid-Based Approaches

Long before full genome sequences for many bacteria were completed, sequences for specific genes of taxonomic importance, particularly 16S rRNA-encoding genes of a wide variety of bacteria, were available. This wealth of data have been used by several researchers to develop new and rapid approaches to profile bacteria. Early studies profiled bacteria with MALDI TOF MS (Hurst et al., 1998) and ESI MS (Muddiman et al., 1996) to determine the size of PCR products of particular marker genes; however, this approach did not provide adequate resolving power to profile bacteria at the strain level.

The discriminatory power of MS-enabled nucleic acid-based approaches to bacterial profiling was increased dramatically by von Wintzingerode et al. (2002), who incorporated a base-cleavage step that generated many more MALDI TOF MS-detectable products of varying size. In this approach, PCR products were first reverse-transcribed to RNA. von Wintzingerode et al. (2002) were able to discriminate 16S rDNA PCR products that differed by only a single nucleotide. Hardware

and software to facilitate this approach are now marketed by Sequenom (San Diego, CA).

Several others have used the highly versatile Sequenom approach to profile a variety of bacteria at the strain level (Lefmann et al., 2004). In a technological tour de force, Honisch et al. (2004) identified mutant strains of *E. coli*, they resequenced 1.54 Mb of the genome of different strains in only 13.5 h. Later, Honisch et al. (2007) used the Sequenom approach to type 100 *Neisseria meningitidis* strains. The group employed publicly available MLST sequence data rather than 16S rRNA sequences, and found that this MALDI-based alternative compared favorably to conventional dideoxy-based sequencing approaches for MLST analyses. More recently, the Sequenom approach has been employed to detect single nucleotide polymorphisms (SNPs) to discriminate among strains of MRSA (Syrmis et al., 2011). High concordance between this MS-based approach and a more conventional method to type MRSA (real-time PCR) suggested that the MS-based approach might be a viable alternative.

A noteworthy feature of nucleic acid-based approaches is that they can be performed on uncultivated microorganisms (von Wintzingerode et al., 2002). This advantage, though, might also represent a disadvantage because contaminating bacteria, either viable or not, can be inadvertently and readily detected with PCR. In addition, more widespread adoption of this approach might be limited by the fact that the requisite hardware and software to perform this type of profiling is quite costly and currently offered by a single vendor.

Nucleic acid-based approaches to profile bacteria at the strain level have been described that do not rely upon the Sequenom technology. Jackson et al. (2007) compared masses of 16S rRNA cleavage products detected with MALDI to more than 47,000 publicly available 16S rRNA sequences in the Ribosome Database Project (Cole et al., 2009) and reported attaining strain-level resolution. In addition, a nucleic acid-based approach to characterize a particularly broad diversity of microorganisms (viruses and fungi in addition to bacteria) with PCR-product characterization has been described, but this approach requires ESI MS rather than MALDI (Ecker et al., 2008).

C. Software

Library-based and bioinformatics-enabled approaches to bacterial profiling at the strain level both involve generation of substantial and complex datasets. Even early work that used MALDI TOF MS to profile bacteria at the strain level underscored the need for software-assisted data analysis (Arnold & Reilly, 1998). Several software solutions have been developed to enhance analysis, and most have focused on the library-based approach to MALDI TOF MS microbial profiling. Bioinformatics-enabled approaches have often relied upon more general purpose software (e.g., MASCOT) for protein identification (Fagerquist et al., 2006). In-house software has been developed, though, to expedite searches and to assign statistical significance to matches (Demirev et al., 2005). Other software (e.g., MATLAB) has also been used to facilitate protein identification during bacterial strain profiling with MALDI (Lasch et al., 2010).

Early efforts to profile strains of bacteria with MALDI TOF MS often relied upon software developed in-house. Arnold and Reilly (1998) described one of the first uses of custom software to facilitate bacterial strain identification with MALDI TOF MS. The rather simple software imported pairs of profiles to be considered. The files from each profile consisted of lists of masses and the corresponding peak intensity of each mass. The software compared the profiles with a modified cross-correlation method that focused on subtle differences between the profiles. Bright et al. (2002) also recognized the importance to analyze the entire MALDI TOF MS profile rather than merely unique and/or particularly intense peaks. The authors described the development of custom software (Manchester Metropolitan University Search Engine; MUSE^{TM}) that allowed the construction and search of libraries of MS profiles of bacterial strains. Leuschner et al. (2003) developed software to rapidly identify consensus peaks present in spectra of *Salmonella enterica* subsp. *enterica* serovars. The software also included a precision measurement that allowed specification of how similar in mass two peaks from different strains must be in order to be considered the same peak. Similarly, Siegrist et al. (2007) used software developed in-house to enhance precision and to perform data reduction by specifying a parameter, w, that represented a mass window width through which a single peak was used to represent all peaks in that window.

Although successes with in-house/custom software to enhance strain-level profiling are common in the literature, it should be noted that single, seemingly minor, software features can have profound effects on the ability of MALDI TOF MS to resolve bacterial strains. Several software algorithms have included peak intensity in calculations that compare spectra (Lundquist et al., 2005; Vargha et al., 2006; Robbins et al., 2007; Dieckmann et al., 2008; Giebel, Fredenberg, & Sandrin, 2008), whereas other algorithms have employed a binary peak list (e.g., those that contain only the presence or absence of peaks; Siegrist et al., 2007; Schmidt et al., 2009; Karger et al., 2010). For example, Robbins et al. (2007) reported that use of a similarity coefficient that included peak intensity (Pearson product–moment correlation coefficient) increased the accuracy of categorization of environmental strains of *E. coli* by 28% in comparison to use of a similarity coefficient that considered only peak presence or absence (Dice similarity coefficient).

A variety of commercially available, general-purpose software packages have been used in combination with one another to facilitate rigorous, quantitative analysis of MALDI TOF MS profiles of bacterial strains (Rupf et al., 2005; Donohue et al., 2006). Rupf et al. (2005) used MATLAB (MathWorks; Natick, MA) and Microsoft Excel (Microsoft Corporation, Redmond, WA) to analyze profiles via multivariate statistical analysis. The approach was quantitative, and specified a threshold parameter (σ_1) for two profiles to be considered unique (Fig. 7). As others noted previously (Arnold & Reilly, 1998; Bright et al., 2002), visual inspection of spectra was not adequate to obtain accurate strain differentiation. The authors noted that individual sum spectra did not yield strain-level resolution (Arnold & Reilly, 1998; Bright et al., 2002). Instead, collections of replicate spectra of each strain ("master spectra") were required. Hettick et al. (2006) also employed rigorous, software-assisted analysis of MALDI TOF MS profiles to obtain reliable strain differentiation. The authors used the open-source statistical programming environment, R (Hornik

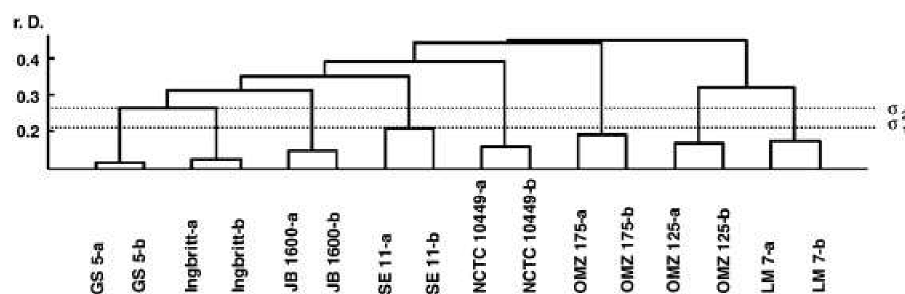

FIGURE 7. A quantitative approach to establishing a threshold parameter, σ, which facilitated reliable strain-level characterization. The maximum distance of replicate profiles (duplicates) is represented by σ_1, while the minimum distance between profiles of different strains is represented by σ_2. Adapted from Rupf et al. (2005).

& Leisch, 2005), to perform random forest analysis of MS profiles to obtain a very low error of strain differentiation. Linear discriminant analysis was performed with SAS (SAS Institute, Inc., Cary, NC). Different strains exhibited common peaks. As such, no strain-specific biomarkers were detected. Analysis of the differential expression of common peaks, though, allowed strain-level resolution. Most recently, Kraushaar et al. (2011) used a suite of R software tools (packages), including those that facilitated data reduction, biomarker identification, and cluster analysis, to enhance categorization of strains of *Y. enterocolitica*.

As with all library-based approaches, a library (or database) of profiles must be constructed. Carbonnelle et al., (2007) used the open-source BGP database software (http://sourceforge.net/projects/bgp) to perform strain identification within the genus *Staphylococcus*. Similarly, the freely available MS Screener (http://web.mpiib-berlin.mpg.de/pdbs/2d-page/downloads.html) was used to facilitate strain-level profiling of *Lactobacillus* (Schmidt et al., 2009). Given the current utility and ubiquity of the library-based approach, it has been suggested that a publicly available, dynamic database of MALDI TOF MS profiles similar to CDC's Pulsenet (Swaminathan et al., 2001), which houses PFGE profiles, be created (Mott et al., 2010).

Although many in-house software solutions have been developed to facilitate strain-level profiling, several commercial software packages have also been developed to enhance analysis of MALDI TOF MS profiles of bacteria. BioTyper (Bruker Daltonics) and the Spectral Archive and Microbial Identification System, SARAMIS (bioMérieux), have emerged as two of the most commonly employed commercially available software solutions. Although the majority of applications that use software-assisted data analysis have focused on species-level characterization (Stackebrandt, Päuker, & Erhard, 2005; Vanlaere et al., 2008; Ilina et al., 2009; Stephan et al., 2010; Benagli et al., 2011; Gaia, Casati, & Tonolla, 2011), several studies suggest that software has the potential to enhance bacterial profiling at the strain level.

Commercially available software packages that facilitate construction of strain-profile databases and query of those databases have grown in popularity. Majcherczyk et al. (2006) used MicrobeLynx software [Waters Corp (Micromass Ltd.), Manchester, UK] to distinguish among strains of MRSA-resistant and susceptible to teicoplanin. Use of this software to facilitate profiling bacteria at the strain level has not been frequent. Of the studies reviewed here, only five used the MicrobeLynx software (Du et al., 2002; Keys et al., 2004; Krader & Emerson, 2004; Majcherczyk et al., 2006; Rajakaruna et al., 2009). Other library-based software packages, including BioTyper and SARAMIS, have been used more frequently. The BioTyper software provides a database environment and algorithm that allows for quantitative comparison and identification of bacteria within the database. Currently, the database contains spectra of 3,025 bacteria and can be extended using in-house acquired spectra. Sauer et al. (2008) reported the first use of the BioTyper software package (Bruker Daltonics) to perform strain identification of *Erwinia* sp. Routine methods specified by the BioTyper product afforded discrimination at the species level, but sub-species resolution required implementation of a weighted pattern-matching approach (described above). Grosse-Herrenthey et al. (2008) also used BioTyper to profile strains of *Clostridia*. Despite the fact that many species within the genus are closely related and difficult to distinguish with existing methods, the authors resolved this group at the strain level with BioTyper. Results of some studies suggest that BioTyper could be improved with regard to its ability to facilitate strain-level resolution. Ayyadurai et al. (2010) used the software to characterize 39 strains of *Yersinia* across 12 species. The software unambiguously identified at the species level, but additional software (ClinProTools; Bruker Daltonics) was used to differentiate *Y. pestis* strains at the biotype level. Specifically, the in-build, quick classifier and genetic algorithms contained within ClinProTools were used to characterize the three biotypes. Similarly, Karger et al. (2010) supplemented use of Biotyper with R to perform rigorous data-reduction strategies that enhanced categorization of strains of shiga toxin-producing *E. coli*.

bioMérieux's SARAMIS represents an alternative software solution to Bruker's BioTyper. Like BioTyper, SARAMIS allows users to construct a custom database of spectra or to purchase a database of spectra from the software manufacturer. Profiles of unknown bacteria are compared to those in the database to characterize the unknown bacterium. Both products allow bacteria of interest to be represented by multiple, composite spectra. BioTyper refers to these spectra as main spectral projections (MSPs), whereas SARAMIS uses the term SuperSpectrum^{TM}. Success with SARAMIS to profile at the strain level has been reported (Dieckmann et al., 2008; Hahn et al., 2011). For example, Stephan et al. (2011) reported use of the software to subtype 117 *Y. enterocolitica* strains with respect to their appropriate biotype. The performance of

Biotyper and SARAMIS have been directly compared (Cherkaoui et al., 2010). Both products were very effective at the species level; each yielded rates of correct identification that exceeded 99%. Unfortunately, no similar side-by-side comparison at the strain level has yet been performed, but it appears both products might require additional development to attain similar levels of performance at the strain level because applications of both products at the strain level have been reported to require additional analysis (e.g., the weighted pattern matching employed; Sauer et al., 2008). In addition, the observation that the limits of taxonomic resolution of MALDI TOF MS profiling might vary across different bacteria suggests that bacterium-specific subroutines or modules will need to be developed and incorporated into the software (Ghyselinck et al., 2011).

III. CHALLENGES AND LIMITS OF CURRENT APPROACHES

Although considerable progress has been made to profile bacteria at the strain level with MALDI TOF MS, the approach faces several impediments to its broader and more widespread application. Library-based approaches have been limited by the fact that no single universal culture condition and sample preparation protocol has been widely adopted. Several have been proposed (Liu et al., 2007; Freiwald & Sauer, 2009). As described in an excellent review (Šedo, Sedláček, & Zdrahal, 2011) of sample-preparation methods used in MALDI profiling of bacteria, the use of diverse sample-preparation techniques has resulted in several discrepancies in the literature regarding the taxonomic limits. The reproducibility of the approach has also limited its broader application at the strain level. Reproducibility must be particularly high when comparing MS profiles of closely related bacteria that yield highly similar profiles. Similarity between replicates of the same bacterium (i.e., reproducibility) must exceed the similarity of profiles of closely related strains of bacteria. Unfortunately, very few studies (e.g., Ghyselinck et al., 2011) have rigorously quantified reproducibility. In addition, although MS profiles are often obtained via automation to leverage the high-throughput potential of MALDI TOF MS, consequent effects of automation on profile reproducibility and quality have only recently been explored (Schumaker, Borror, & Sandrin, 2012).

Bioinformatics-enabled approaches to MS profiling at the strain level might obviate many limitations associated with library-based approaches (Dieckmann et al., 2008); however, these approaches are limited by the fact that they tend to: (1) have significantly greater hardware and software requirements (i.e., top-down approaches, in particular, typically require more sophisticated and expensive TOF-TOF or other MS-MS capable instruments); (2) require more time, labor, and training (particularly those approaches that rely upon sample digestion and pre-fractionation); and (3) work best with bacteria for which complete genome sequences are available, although success with characterization on unsequenced bacteria with MALDI TOF MS (Teramoto et al., 2009; Fox et al., 2011) and other MS approaches, including an Orbitrap (Wynne et al., 2009; Wynne, Edwards, & Fenselau, 2010) have been described. While the requisite hardware and software to perform bioinformatics-enabled profiling at the strain level becomes more readily available and economical, these approaches may become more common. In addition, novel approaches to characterize bacteria without fully sequenced genomes as well as the ever-increasing number of bacteria with fully sequenced genomes will certainly facilitate more widespread use of bioinformatics-enabled approaches as has been suggested previously (Demirev & Fenselau, 2008a). Considering the more widespread application of library-based approaches to profile bacteria at the strain level, the following sections focus on challenges and limits associated with library-based approaches.

A. Sample Preparation and Culture Conditions

Broadly speaking, two approaches to prepare samples for analysis with library-based approaches have been employed: intact cell-based methods and cell extract-based methods (Tables 1–3). Intact cell-based methods place suspensions of intact cells on the MALDI target (Stackebrandt, Päuker, & Erhard, 2005; Moura et al., 2008; Schumaker, Borror, & Sandrin, 2012), whereas cell extract-based methods place cell extracts (supernatants) alone on the target (Vargha et al., 2006; Schmidt et al., 2009; Fujinami et al., 2011). Closer examination of sample-preparation methods reveals substantial variability in sample preparation (Šedo, Sedláček, & Zdráhal, 2011). Important sample-preparation features, including choice of matrix, have varied considerably (Tables 1–3). In many cases, multiple preparation methods were explored as an approach to maximize the taxonomic resolution of the method (Williams et al., 2003; Ruelle et al., 2004; Liu et al., 2007; Dieckmann et al., 2008); however, in other cases, a rationale to use a particular method was not provided. Interestingly, commercially available software products recommend different cell-preparation approaches. bioMérieux's SARAMIS recommends direct deposition of cells onto the MALDI target, whereas Bruker's BioTyper suggests a cell extract-based sample-preparation approach.

Most applications of MALDI TOF MS profiling of bacteria at the strain level have used cell extract-based sample preparation methods (Tables 1–3). In fact, one of the first reports of strain-level resolution employed a sample-preparation method that relied upon cell extracts (Krishnamurthy, Rajamani, & Ross, 1996). The authors used chemical and mechanical lysis techniques to prepare extracts that yielded strain-specific biomarkers of members of the genus *Bacillus*. Several subsequent studies reported that cell extract-based sample-preparation methods had also allowed strain-level resolution. For example, Nilsson (1999) reported strain-level resolution of *H. pylori* with cell extract-based sample-preparation methods. Perhaps not surprisingly, the authors noted that the choice of extraction solvent (either ACN/water or 0.1% TFA) dramatically affected which strain-specific biomarkers were detected. The authors also reported dramatic effects of matrix on peak detection. Horneffer et al. (2004) used wet-heat treatment to extract additional analytes to facilitate strain-level resolution of *B. subtilis* and *B. cereus*. Others have used enzymatic pretreatment to facilitate more complete extraction of cell contents, often with trypsin (Krishnamurthy, Rajamani, & Ross, 1996) or lysozyme (Vargha et al., 2006; Giebel, Fredenberg, & Sandrin, 2008). More recently, Fujinami et al. (2011) relied upon cell extract-based sample-preparation methods to differentiate strains of *Legionella*. The authors used membrane-

filtered cell extracts obtained by exposing cells to 1% TFA. This extraction approach was compared to one that involved bead beating with zirconia/silica beads. The membrane-filtered extracts yielded more useful strain-discriminating peaks than the extracts obtained with bead beating. Although there is no clear consensus on a universal cell extract-based sample-preparation method, an ethanol–formic acid extraction procedure (Sauer et al., 2008) has been recently employed by many (Barbuddhe et al., 2008; Ayyadurai et al., 2010; Dubois et al., 2010; Wensing, Zimmermann, & Geider, 2010; Ghyselinck et al., 2011).

Although strain-level resolution has been reported by many groups with cell extract-based sample-preparation methods, intact cell-based methods have also shown promise. In addition, intact cell-based preparation approaches are simpler and more rapid, because they do not require additional steps involved in chemical, enzymatic, and mechanical extraction often associated with cell extract preparation (Freiwald & Sauer, 2009). Several studies have reported strain-level resolution by depositing cells directly on the MALDI target. It should be noted that, although many studies placed intact cells directly onto MALDI targets and subsequently overlaid them with matrix and solvent mixtures (Walker et al., 2002; Jackson et al., 2005; Carbonnelle et al., 2007; Siegrist et al., 2007; Dieckmann et al., 2008), others suspended intact cells in matrix/solvent (often TFA and ACN) mixtures prior to deposition onto the target (Arnold & Reilly, 1998; Welham et al., 1998; Ryzhov, Hathout, & Fenselau, 2000; Dickinson et al., 2004; Donohue et al., 2006; Moura et al., 2008). Both approaches to intact cell-based sample preparation have yielded strain-level resolution; however, very few rigorous quantitative comparisons between direct deposition and suspension prior to deposition have been reported in the literature. Jackson et al. (2005), in their efforts to develop a standardized approach to profile MRSA at the strain level, presented a comparison of intact cell deposition (as colonies from agar plates) to deposition of suspended cells (in matrix solvent or water) onto the target. The authors found that direct deposition of strains from agar plates onto the MALDI target was more effective than suspending cells prior to deposition. Directly deposited cells yielded higher quality spectra that exhibited higher reproducibility, numbers of peak per profile, signal intensity, and S:N (Fig. 8).

Despite extensive efforts to optimize a set of standard sample-preparation methods (Jackson et al., 2005; Liu et al., 2007), most attempts to profile MRSA and other bacteria at the strain level with MALDI TOF MS have not used standardized sample-preparation protocols. This lack of broad and consistent implementation of a standardized approach has resulted in conflicting reports in the literature. For example, inconsistencies in the literature on the ability of MALDI TOF MS to discriminate between strains of methicillin-resistant and methicillin-sensitive strains of *S. aureus* are common. Several studies reported that MALDI TOF MS can resolve strains of methicillin-resistant and methicillin-sensitive strains of *S. aureus* (Edwards-Jones et al., 2000; Du et al., 2002; Majcherczyk et al., 2006), whereas others have not reported such success (Bernardo et al., 2002; Walker et al., 2002; Jackson et al., 2005). These divergent results might have been caused, at least in part, by different sample-preparation methods.

As with sample-deposition methods, culture conditions have varied considerably across efforts to profile bacteria at the strain level with MALDI TOF MS. A variety of microbiological media, including solid agar and broth types, have been employed. The type of microbiological medium used has been widely reported to affect MS profiles of bacteria (Walker et al., 2002; Ruelle et al., 2004; Valentine et al., 2002), but some have suggested that effects of medium type on spectra are subtle and do not affect the overall ability of MALDI TOF MS to discriminate among bacteria (Conway et al., 2001; Bernardo et al., 2002; Vargha et al., 2006). Conway et al. (2001) reported that profiles of *E. coli* grown in two different broths exhibited 80% similarity. Given that strain-level profiling is more sensitive to minor differences in spectra associated with strain differences, the importance of an appropriate and consistent medium is likely far more important in strain-level

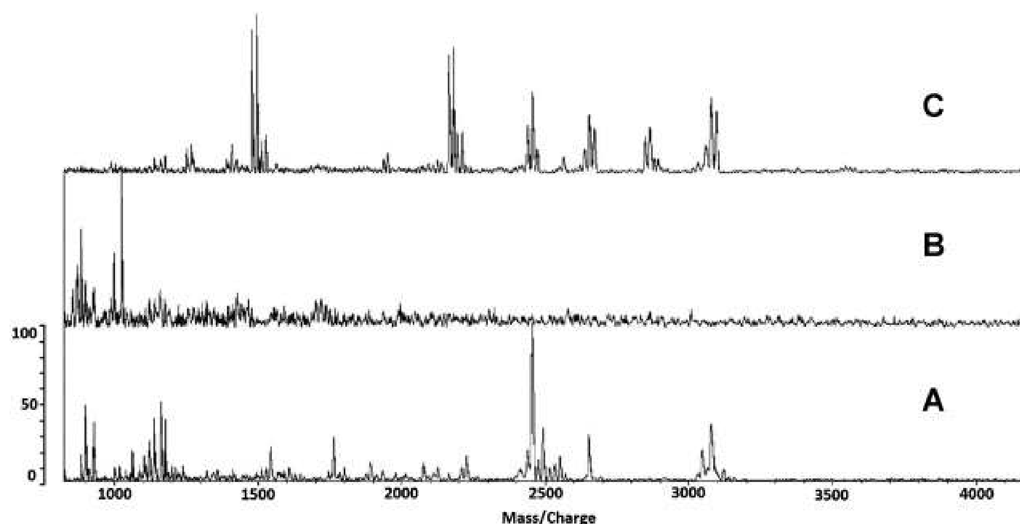

FIGURE 8. Direct deposition of cells of MRSA onto the MALDI target (**A**) yielded spectra of higher quality than deposition of cells suspended in matrix solvent (**B**) or water (**C**). Adapted from Jackson et al. (2005).

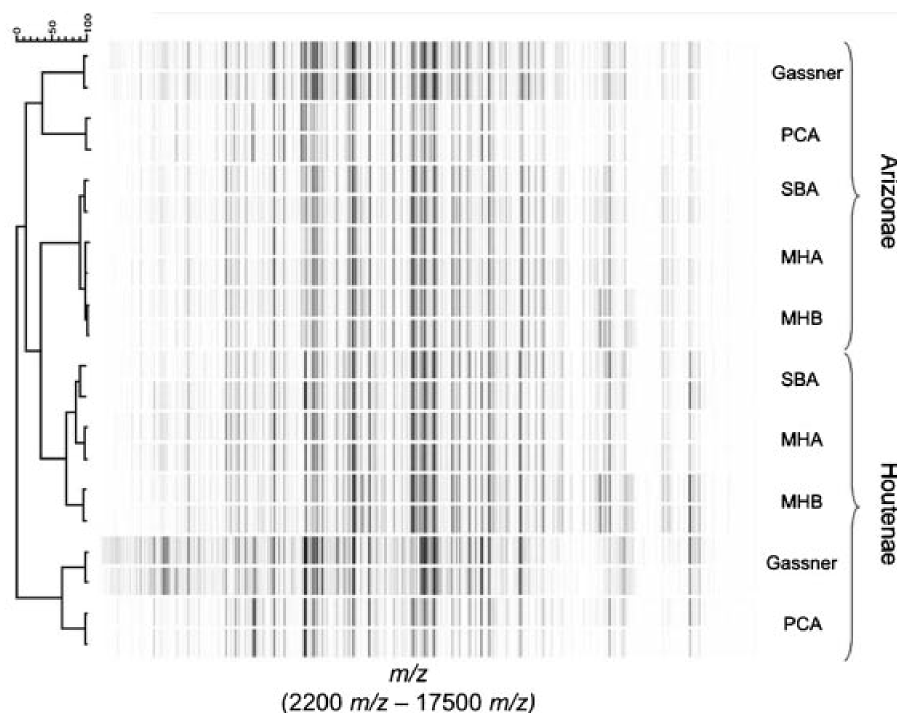

FIGURE 9. Medium type affected profiles of *Salmonella enterica* subspecies, but these effects did not prevent grouping of strains with respect to subspecies (Arizonae or Houtenae). Duplicate spectra are represented in a gel view format in which peaks and their respective intensities are represented by grayscale bands. PCA, plate count agar; SBA, sheep blood agar; MHA, Mueller–Hinton agar; MHB, Mueller–Hinton blood agar. Adapted from Dieckmann et al. (2008).

applications than in species-level applications. For example, Horneffer, Haverkamp, and Janssen (2004) noted significant effects of medium type on MS profiles of spores of strains of *B. subtilis*. Rupf et al. (2005) attributed their success to obtaining strain-level resolution of mutans streptococci, in part, to their use of strictly controlled culture conditions. Similarly, in their efforts to discriminate strains of *S. pyogenes*, Moura et al. (2008) reported that medium type (blood agar or THB) affected MALDI profiles. Dieckmann et al. (2008) also noted medium-specific profiles and medium-dependent clustering (Fig. 9), but concluded that strain-specific biomarker peaks were mostly consistent across multiple media. In addition, such subtle changes did not prevent representatives of two subspecies, *arizonae* and *houtenae*, from clustering together. Similarly, Sauer et al. (2008) used media (Luria Broth) supplemented with one of two carbon sources, glycerol or glucose. Neither supplement had a significant effect on MS profiles or the ability to identify individual strains. Grosse-Herrenthey et al. (2008) reported similar, largely insignificant, effects of medium type on profiling *Clostridia* at the strain level. Taken together, differing results from these studies suggest that medium type has the potential to affect MALDI TOF MS profiles, but these effects might be bacterium-specific and limited. Potential effects should be thoroughly investigated and quantified before medium type is varied.

In addition to culture-medium type, culture conditions (whether cells were grown on solid agar or in broth) have also been varied across attempts to profile bacteria at the strain level with MALDI. In many cases, cells cultured on solid media have been used (Rajakaruna et al., 2009; Pennanec et al., 2010). In others, broth cultures have been used (Moura et al., 2008; Schumaker, Borror, & Sandrin, 2012). Greater cell heterogeneity associated with plate cultures might explain these observations. Bacterial colonies consist of cells that vary in age, with the oldest cells found in the center of the colony and the newest colonies at the perimeter. In contrast, broth cultures tend to contain more homogenous populations of cells that are synchronized in their growth (Madigan et al., 2009). Additional research is warranted to determine the extent to which culture conditions affect MALDI TOF MS profiling of bacteria at the strain level. In addition, possible interactions among sample preparation approach (intact cells or extract), medium type, and culture conditions warrant investigation.

B. Reproducibility

The assessment of effects of sample preparation and culture conditions as well as overall method reliability and efficacy requires rigorous quantification of reproducibility. Such assessment is particularly true at the strain level because often only very minor differences in MS profiles facilitate resolution of closely related bacterial strains. Unfortunately, no standardized approach to quantify and report reproducibility has been widely implemented. In addition, the term reproducibility has been used with different meaning by different authors. Rather infrequently, reproducibility has been used to refer to the accuracy with which strains were identified (Mellmann et al., 2009). Most commonly and in this review, though, reproducibility

refers to how similar (or different) replicate spectra of the same strain are to one another with regard to peak presence/absence and often peak intensity (Giebel, Fredenberg, & Sandrin, 2008; Moura et al., 2008; Pennanec et al., 2010).

Several studies have reported reproducibility based upon visual inspection of spectra (Arnold & Reilly, 1998; Jackson et al., 2005; Lui et al., 2007; Wolters et al., 2011). Several efforts to more rigorously quantify reproducibility relied upon the root mean square (RMS) of replicate spectra (Keys et al., 2004; Majcherczyk et al., 2006; Rajakaruna et al., 2009). Freiwald & Sauer (2009) described the coefficient of variation (CV) of replicate spectra, but this approach requires specialized and proprietary software (ClinProTools; Bruker Daltonics). An alternative approach, not reliant upon specialized software (only MATLAB), that employed a combined analysis of variance (ANOVA)-principal component analysis (PCA) has also been proposed, but not widely adopted (Chen, Lu, & Harrington, 2008). Our group has quantified reproducibility using similarity coefficients of spectra calculated using the following Pearson correlation coefficient (r) in which x_i and y_i represent intensity values of peaks in two profiles, x and y, and n represents the number of peaks in each profile (Giebel, Fredenberg, & Sandrin, 2008; Devore, 2012; Schumaker, Borror, & Sandrin, 2012):

$$r = \frac{\sum_{i=1}^{n} x_i y_i - \frac{1}{n}\sum_{i=1}^{n} x_i \sum_{i=1}^{n} y_i}{\sqrt{\sum_{i=1}^{n} x_i^2 - \frac{1}{n}\left(\sum_{i=1}^{n} x_i\right)^2}\sqrt{\sum_{i=1}^{n} y_i^2 - \frac{1}{n}\left(\sum_{i=1}^{n} y_i\right)^2}}$$

As with another approach (Freiwald & Sauer, 2009), though, these analyses are facilitated by proprietary and rather specialized software (BioNumerics; Applied Maths; Sint-Martens-Latem, Belgium). Indeed, a standard approach to quantify reproducibility that uses tools widely available to investigators will be required to enable future meaningful comparisons of reproducibility reported by different studies.

Although relatively few studies have rigorously quantified reproducibility, even fewer have implemented measures to ensure that reproducibility was at an appropriate threshold level to allow reliable resolution of bacterial strains. In this regard, the work by Rupf et al. (2005) is exemplary in its approach to quantify threshold parameters, σ_1 and σ_2, which quantify the similarity of replicate spectra and spectra from different strains, respectively, as described above in Section II.C. (Fig. 7). Broad implementation of a similar approach seems critically important to demonstrate the reliability of library-based methods to profile bacteria at the strain level.

Rigorous and quantitative assessments of reproducibility will likely be critically important as applications of MALDI TOF MS-based profiling of bacteria at the strain level rely more upon automated data acquisition. Automated data acquisition has been proposed to increase reproducibility in comparison to manual data acquisition, in which different operators collect spectra (Freiwald & Sauer, 2009). Most modern mass spectrometers are bundled with instrument operation software that facilitates fully automated acquisition of spectra. Instrument operators need only to specify minimum spectrum quality criteria (e.g., base peak resolution, S:N, etc.). As such, automated data acquisition should facilitate more objective acquisition than manual acquisition by a human operator. The potential benefits of automated data acquisition to throughput and reproducibility have made this type of data collection common (Seng et al., 2009; Cherkaoui et al., 2010; van Veen, Claas, & Kuijper, 2010; De Bruyne et al., 2011). A recent report, though, suggests that data-acquisition automation can reduce MS profile quality and reproducibility (Schumaker, Borror, & Sandrin, 2012; Fig. 10). Reproducibility was lower when spectra were acquired using automation (Fig. 10A) than when spectra were acquired by either an experienced (Fig. 10B) or a more novice (Fig. 10C) operator. These findings suggest that effects of automation on reproducibility must be considered, particularly at the strain level, where minor differences in profiles can have profound effects on the ability of the method to reliably resolve closely related bacterial strains. In addition, efforts should be made to ensure that parameters used in automation maximize reproducibility and spectrum quality.

IV. FUTURE DIRECTIONS

A. Non-Protein-Based Approaches

Although the majority of non-protein-based approaches to MALDI TOF MS profiling at the strain level have focused on analysis of nucleic acids (Hurst et al., 1998; von Wintzengrode et al., 2002; Honisch et al., 2004; Syrmis et al., 2011), few have focused on lipids and lipopeptides that are observed in a strain-specific manner (Leenders et al., 1999; Mukherjee & Das, 2005). Leenders and colleagues used MALDI TOF MS to observe and characterize structurally (via PSD) lipopeptides produced by *B. subtilis* in a strain-specific manner (Leenders et al., 1999). Similarly, Price et al. (2007) detected strain-resolving lipopeptides in *B. subtilis* strains obtained from diverse geographic locations (Fig. 11). A more recent study used lipid profiles to discriminate bacteria with MALDI, but strain-level resolution was not reported (Calvano, Zambonin, & Palmisisano, 2011). Although lipid and lipoprotein-based approaches to characterize bacteria at the strain level appear to have promise, application of this approach across greater numbers of closely related bacteria might require more rigorous structural lipid characterization and discrimination than is typically obtained with MALDI TOF MS. For example, Zhang et al. (2011) recently reported characterization of fatty acids with low-temperature plasma (LTP) MS that facilitated differentiation of strains of *Salmonella enterica*.

B. Immunological and Lectin-Based Approaches

Specificity is of paramount importance to all strain-level applications of microbial identification and characterization. Immunological (e.g., antibodies) and lectin-based chemistries have shown promise to enhance the sensitivity and specificity of MALDI TOF MS-based approaches to characterize bacteria. Early efforts focused on increasing method sensitivity. Bundy and Fenselau (2001) used non-immune chemistry (carbohydrate-binding lectins) to enhance detection of bacteria in urine, milk, and processed chicken. A similar approach was later employed to detect *B. cereus* and *E. coli* (Afonso & Fenselau, 2003). Madonna et al. (2001) used polyclonal antibodies with greater specificity than lectins to detect *Salomonella choleraesuis* from bacterial mixtures and from river water, urine, and blood to which the bacterium had been added. Madonna and colleagues later employed a similar approach with

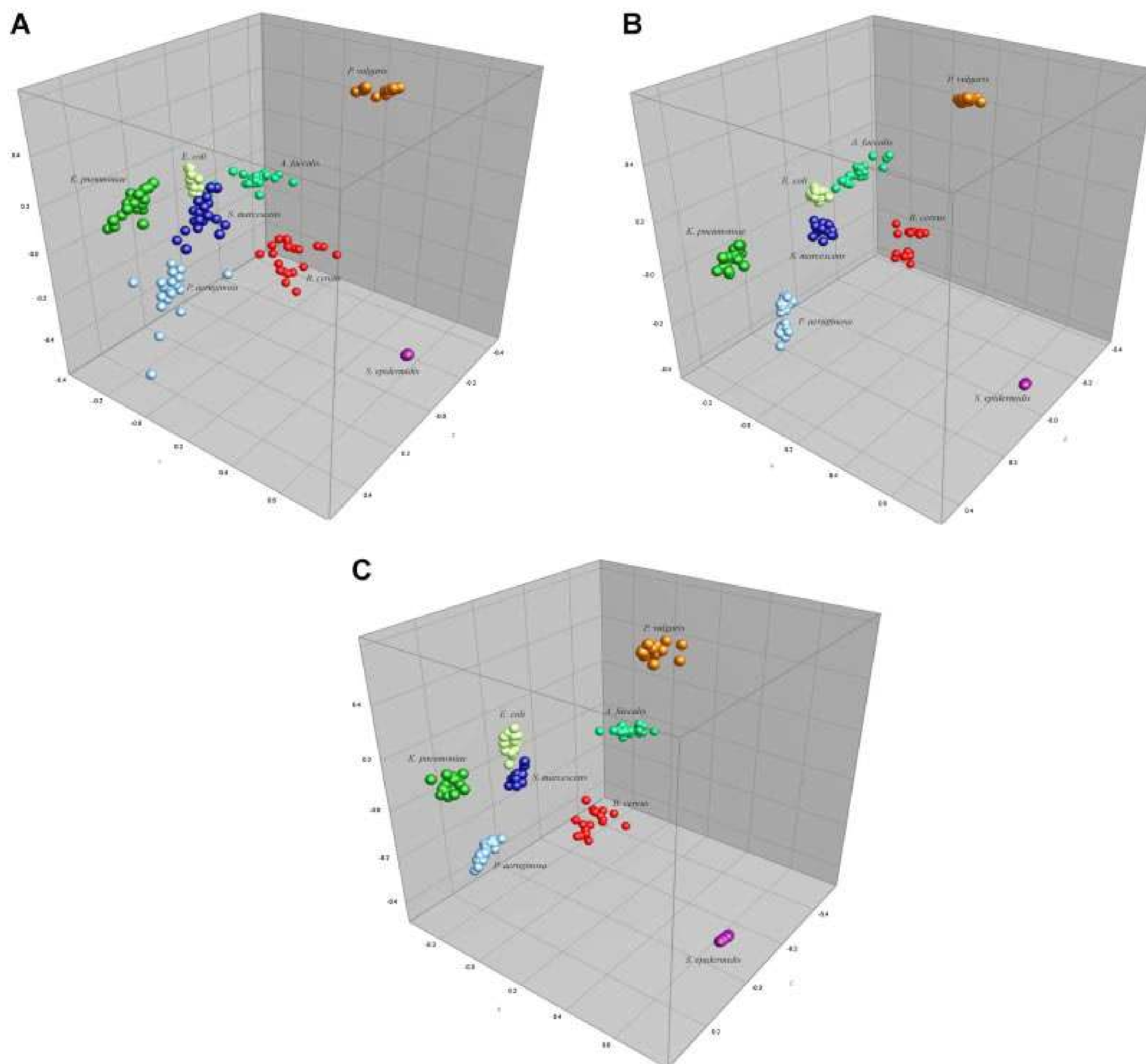

FIGURE 10. Mode of data acquisition affected reproducibility of MALDI-MS profiles of several bacteria. Similarities of replicate spectra acquired via automation (**A**), an operator with 2 years of experience (**B**), and an operator with 6 months of experience (**C**) are represented by multidimensional scaling (MDS) analysis. Adapted from Schumaker, Borror, and Sandrin (2012).

immunomagnetic separation and phage amplification to reduce the detection threshold of the method from 1×10^6 to 5×10^4 cells/mL (Madonna, Cuyk, & Voorhees, 2003). The same authors have asserted that MALDI TOF MS-based immunological methods can help overcome limitations commonly associated with more conventional detection approaches (e.g., ELISA, dot blot assays, etc.) that include production of results that are: (1) exclusively positive/negative (binary) and (2) falsely positive (Madonna, Cuyk, & Voorhees, 2003). Ochoa and Harrington (2005) developed a strain-specific approach that used antibodies specific for the O157:H7 strain of *E. coli*. More recently, effective antibody-based approaches have been described that rely upon technologies other than MALDI, such as FT-IR spectroscopy (De Lamo-Castellvi, Männing, & Rodríguez-Saona, 2010). Taken together, results from these studies suggest that immunological and lectin-based approaches provide greater specificity than current methods; however, detection of multiple strains will likely require use of a separate, specific antibody, or a particular lectin for each strain. As such, the cost and time associated with antibody and/or lectin production might limit the utility of these approaches to those applications in which detection of very few strains is desired. Nonetheless, for strains that yield MALDI profiles too similar to distinguish with conventional approaches (Albesharat et al., 2011; Ghyselinck et al., 2011), these approaches might provide the additional resolution required to afford strain-level resolution.

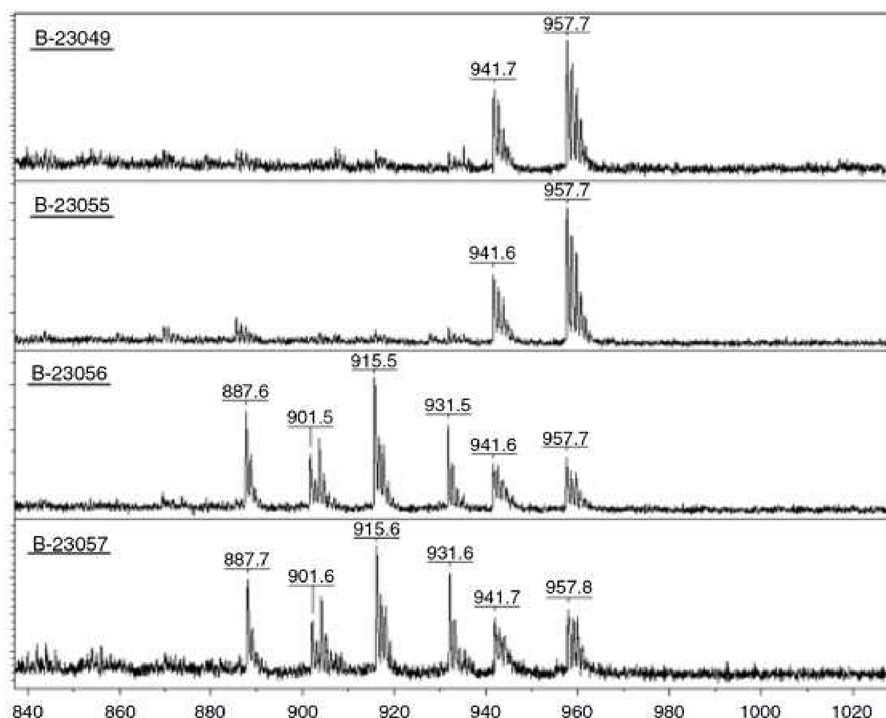

FIGURE 11. MALDI-MS analysis of lipopeptides produced in a strain-specific manner by four desert strains of *Bacillus subtilis*. Adapted from Price et al. (2007).

C. Characterization of Metabolic State

Although several researchers have concluded that the limits of the taxonomic resolution of MALDI TOF MS profiling of bacteria is at or near the strain level (Rezzonico et al., 2010; Gaia, Casati, & Tonolla, 2011), two recent studies suggest that this conclusion might be an underestimate. Kuehl et al. (2011) recently reported MALDI TOF MS profiling in conjunction with multivariate data analyses previously reported for routine strain-based applications (Vargha et al., 2006) to discriminate between different metabolic states of *E. faecalis*. With a simple intact cell sample-preparation approach, the authors obtained profiles of *E. faecalis* that varied with respect to their metabolic state (viable but not culturable (VBNC), post-reactivation, and exponential growth; Fig. 12). Similar beyond-strain level characterization has also been reported (Shah et al., 2011) with a SELDI-based approach to detect *S. aureus* strains in the midst of a transition to methicillin resistance. These advances suggest that the true limit of the taxonomic resolution of MALDI TOF MS-based profiling of bacteria might not yet have been reached.

V. CONCLUDING REMARKS

Successes with MALDI TOF MS profiling at the species level and above in clinical microbiology, environmental microbiology, and counter-bioterrorism have motivated exploration of the taxonomic limits of resolution of this rapid approach to bacterial characterization. Scores of studies summarized in this review demonstrate that MALDI TOF MS profiling at the strain level is indeed possible. The approach compares favorably with conventional approaches; however, the taxonomic resolution reported in each of these strain-level applications has varied considerably. Several strain-level studies have focused on strain categorization, whereas others have sought higher levels of taxonomic resolution with approaches that facilitated strain differentiation and strain identification. Some of the variability in reports of the taxonomic limits of resolution can likely be ascribed to the recent suggestion that

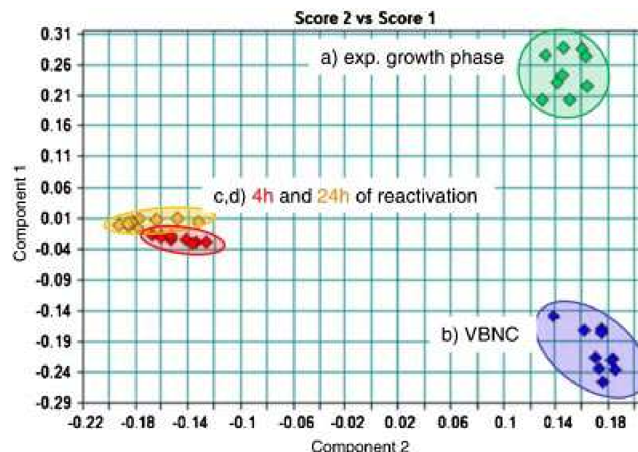

FIGURE 12. The limits of taxonomic resolution of MALDI-MS profiling of bacteria may lie beyond the strain level. Principal component analysis (PCA) of MALDI-MS profiles of a single strain of *Enterococcus faecalis* in different metabolic states. VBNC, viable but non-culturable. Adapted from Kuehl et al. (2011).

taxonomic resolution is taxon-dependent (Ghyselinck et al., 2011). In addition, effects of sample preparation, microbiological medium, instrument, and data analysis approaches have likely influenced reported levels of taxonomic resolution. Undoubtedly, as suggested (Dieckmann et al., 2008), reliable strain-level characterization with MALDI TOF MS will require greater information content, reproducibility, and mass accuracy than characterization at the species level. Although strain-level resolution has been clearly demonstrated for many bacterial species (many of which contain member strains that are remarkably diverse), there are quite likely bacterial species for which strain-level resolution with MALDI TOF MS cannot be obtained (particularly those species that contain member strains that are remarkably similar to one another). For bacteria that fail to yield strain-specific MALDI TOF MS profiles, alternative mass spectrometry technologies, particularly those that provide more rigorous structural biomarker characterization (e.g., ESI-MS), might be required to attain the desired level of taxonomic resolution.

Despite successes of MALDI TOF MS profiling at the strain level, barriers to broader implementation of the approach remain. Fenselau and Demirev (2011) have noted that a publicly available library of profiles would facilitate broader implementation of MALDI TOF MS profiling of bacteria. For strain-level applications, taxon-specific libraries (e.g., a library for environmental strains of *E. coli*) might be required, if one considers that the taxonomic resolution of the method might be taxon specific. Furthermore, strain-specific libraries must contain only spectra that have been acquired under very stringent conditions, including standardized culture conditions, incubation time, sample preparation, sample deposition, data acquisition criteria, and data extraction (e.g., peak-picking). In addition, standard, widely available, open source software to perform data extraction, analysis, and reproducibility measurement will surely advance broader application of MALDI TOF MS profiling at the strain level.

VI. ABBREVIATIONS

1D SDS–PAGE	one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis
2D GE	two-dimensional gel electrophoresis
ACN	acetonitrile
ANOVA	analysis of variance
BGP	bacterial graphic profile
CID	collisionally induced decomposition
CHCA	α-cyano-4-hydroxycinnamic acid
CMBT	5-choloro-2-mercaptobenzothiazole
CoNS	coagulase-negative staphlylococci
CPD	corona plasma discharge
CV	coefficient of variation
DAC	diammonium citrate
DHB	2-hydroxy-5-methoxy benzoic acid
DNA	deoxyribonucleic acid
ESI MS	electrospray ionization mass spectrometry
FA	ferulic acid
FT-IR	Fourier transformation infrared spectroscopy
HABA	2,4-hydroxyphenylazobenzoic acid
HPLC	high-performance liquid chromatography
IBD	inflammatory bowel disease
LTP MS	low-temperature plasma mass spectrometry
m/z	mass-to-charge ratio
MALDI	matrix-assisted laser desorption/ionization
MALDI TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MCA	4-methoxycinnamic acid
MLST	multilocus sequence typing
MRSA	methicillin-resistant *Staphyloccus aureus*
MS	mass spectrometry
MSP	Main Spectra Library
MSSA	methicillin-sensitive *Staphyloccus aureus*
MUSE™	Manchester Metropolitan University Search Engine
RAPD	randomly amplified polymorphic DNA
PCA	principal component analysis
PCR	polymerase chain reaction
PFGE	pulse-field gel electrophoresis
PMF	peptide mass fingerprinting
PSD	post-source decay
RMS	root mean squared
RNA	ribonucleic acid
S:N	signal-to-noise ratio
SA	3,5-dimethoxy-4-hydroxycinnamic acid (sinapininc acid)
SAS	statistical analysis system
SARAMIS	Spectral Archive and Microbial Identification System
SELDI	surface-enhanced laser desorption/ ionization
SIBI	species-identifying biomarker ion
SNP	single nucleotide polymorphism
SSU	small sub unit
TFA	trifluoroacetic acid
THAP	2,4,6-trihydroxyacetophenon
TOF	time-of-flight
THB	Todd–Hewitt broth
VBNC	viable but not culturable
3-HPA	3-hydroxypicolinic acid

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REFERENCES

Afonso C, Fenselau C. 2003. Use of bioactive glass slides for matrix-assisted laser desorption/ionization analysis: Application to microorganisms. Anal Chem 75:694–697.

Albesharat R, Ehrmann MA, Korakli M, Yazaji S, Vogel RF. 2011. Phenotypic and genotypic analyses of lactic acid bacteria in local

fermented food, breast milk and faeces of mothers and their babies. Syst Appl Microbiol 34:148–155.

Anhalt JP, Fenselau C. 1975. Identification of bacteria using mass spectrometry. Anal Chem 47:219–225.

Arnold RJ, Reilly JP. 1998. Fingerprint matching of *E. coli* strains with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of whole cells using a modified correlation approach. Rapid Commun Mass Spectrom 12:630–636.

Arnold RJ, Karty JA, Ellington AD, Reilly JP. 1999. Monitoring the growth of a bacteria culture by MALDI-MS of whole cells. Anal Chem 71:1990–1996.

Ayyadurai S, Flaudrops C, Raoult D, Drancourt M. 2010. Rapid identification and typing of *Yersinia pestis* and other *Yersinia* species by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. BMC Microbiol 10:285.

Bairoch A, Apweiler R. 1996. The SWISS-PROT Protein Sequence Data Bank and its new supplement TREMBL. Nucleic Acids Res 24: 21–25.

Barbuddhe SB, Maier T, Schwarz G, Kostrzewa M, Hof H, Domann E, Chakraborty T, Hain T. 2008. Rapid identification and typing of *Listeria* species by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 74:5402–5407.

Benagli C, Rossi V, Dolina M, Tonolla M, Petrini O. 2011. Matrix-assisted laser desorption ionization-time of flight mass spectrometry for the identification of clinically relevant bacteria. PLoS ONE 6:e16424.

Bernardo K, Pakulat N, Macht M, Krut O, Seifert H, Fleer S, Hünger F, Krönke M. 2002. Identification and discrimination of *Staphylococcus aureus* strains using matrix-assisted laser desorption/ionization-time of flight mass spectrometry. Proteomics 2:747–753.

Böhme K, Fernández-No IC, Gallardo JM, Cañas B, Calo-Mata P. 2010. Safety assessment of fresh and processed seafood products by MALDI-TOF mass fingerprinting. Food Bioprocess Technol 4:907–918.

Böhme K, Fernández-No IC, Barros-Velázquez J, Gallardo JM, Cañas B, Calo-Mata P. 2011. Rapid species identification of seafood spoilage and pathogenic Gram-positive bacteria by MALDI-TOF mass fingerprinting. Electrophoresis 32:2951–2965.

Bright JJ, Claydon MA, Soufian M, Gordon DB. 2002. Rapid typing of bacteria using matrix-assisted laser desorption ionisation time-of-flight mass spectrometry and pattern recognition software. J Microbiol Methods 48:127–138.

Bundy J, Fenselau C. 2001. Lectin-based affinity capture for MALDI-MS analysis of bacteria. Anal Chem 71:1460–1463.

Cain TC, Lubman DM, Weber WJ, Jr. 1994. Differentiation of bacteria using protein profiles from matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 8:1026–1030.

Calvano CD, Zambonin CG, Palmisano F. 2011. Lipid fingerprinting of Gram-positive lactobacilli by intact cells—Matrix-assisted laser desorption/ionization mass spectrometry using a proton sponge based matrix. Rapid Commun Mass Spectrom 25:1757–1764.

Camara JE, Hays FA. 2007. Discrimination between wild-type and ampicillin-resistant *Escherichia coli* by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Anal Bioanal Chem 389:1633–1638.

Carbonnelle E, Beretti JL, Cottyn S, Quesne G, Berche P, Nassif X, Ferroni A. 2007. Rapid identification of Staphylococci isolated in clinical microbiology laboratories by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 45: 2156–2161.

Cassagne C, Ranque S, Normand AC, Fourquet P, Thiebault S, Planard C, Hendrickx M, Piarroux R. 2011. Mould routine identification in the clinical laboratory by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. PLoS ONE 6:e28425.

Castanha ER, Vestal M, Hattan S, Fox A, Fox KF, Dickinson D. 2007. *Bacillus cereus* strains fall into two clusters (one closely and one more distantly related) to *Bacillus anthracis* according to amino acid substitutions in small acid-soluble proteins as determined by tandem mass spectrometry. Mol Cell Probes 21:190–201.

Chen P, Lu Y, Harrington PB. 2008. Biomarker profiling and reproducibility study of MALDI-MS measurements of *Escherichia coli* by analysis of variance-principal component analysis. Anal Chem 80:1474–1481.

Cherkaoui A, Hibbs J, Emonet S, Tangomo M, Girard M, Francois P, Schrenzel J. 2010. Comparison of two matrix-assisted laser desorption ionization-time of flight mass spectrometry methods with conventional phenotypic identification for routine identification of bacteria to the species level. J Clinl Microbiol 48:1169–1175.

Claydon MA, Davey SN, Edwards-Jones V, Gordon DB. 1996. The rapid identification of intact microorganisms using mass spectrometry. Nat Biotechnol 14:1584–1586.

Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM, Marsh T, Garrity GM, Tiedje JM. 2009. The Ribosomal Database Project: Improved alignments and new tools for rRNA analysis. Nucleic Acids Res 37:D141–D145.

Colquhoun DR, Schwab KJ, Cole RN, Halden RU. 2006. Detection of norovirus capsid protein in authentic standards and in stool extracts by matrix-assisted laser desorption ionization and nanospray mass spectrometry. Appl Environ Microbiol 72:2749–2755.

Conway G, Smole S, Sarracino D, Arbeit R, Leopold P. 2001. Phyloproteomics: Species identification of *Enterobacteriaceae* using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J Mol Microbiol Biotechnol 3:103–112.

Dai Y, Li L, Roser DC, Long SR. 1999. Detection and identification of low-mass peptides and proteins from solvent suspensions of *Escherichia coli* by high performance liquid chromatography fractionation and matrix-assisted laser desorption/ionization mass spectrometry. Rapid Commun Spectrom 13:73–78.

De Bruijn FJ. 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl Environ Microbiol 58:2180–2187.

De Bruyne K, Slabbinck B, Waegeman W, Vauterin P, De Baets B, Vandamme P. 2011. Bacterial species identification from MALDI-TOF mass spectra through data analysis and machine learning. Syst Appl Microbiol 3:20–29.

De Lamo-Castellví S, Männing A, Rodríguez-Saona LE. 2010. Fourier-transform infrared spectroscopy combined with immunomagnetic separation as a tool to discriminate *Salmonella* serovars. Analyst 135:2987.

Demirev PA, Fenselau C. 2008a. Mass spectrometry for rapid characterization of microorganisms. Annu Rev Anal Chem 1:71–93.

Demirev PA, Fenselau C. 2008b. Mass spectrometry in biodefense. J Mass Spectrom 43:1441–1457.

Demirev PA, Ho YP, Ryzhov V, Fenselau C. 1999. Microorganism identification by mass spectrometry and protein database searches. Anal Chem 71:2732–2738.

Demirev PA, Feldman AB, Kowalski P, Lin JS. 2005. Top-down proteomics for rapid identification of intact microorganisms. Anal Chem 77:7455–7461.

Devore J. 2012. Probability and statistics for engineering and the sciences. 8th edition. Boston: Brooks/Cole. 768 p.

Dickinson DN, La Duc MT, Haskins WE, Gornushkin I, Winefordner JD, Powell DH, Venkateswaran K. 2004. Species differentiation of a diverse suite of *Bacillus* spores by mass spectrometry-based protein profiling. Appl Environ Microbiol 70:475–482.

Dieckmann R, Helmuth R, Erhard M, Malorny B. 2008. Rapid classification and identification of *Salmonellae* at the species and subspecies levels by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 74:7767–7778.

Donohue MJ, Smallwood AW, Pfaller S, Rodgers M, Shoemaker JA. 2006. The development of a matrix-assisted laser desorption/ionization mass spectrometry-based method for the protein fingerprinting and identification of *Aeromonas* species using whole cells. J Microbiol Methods 65:380–389.

Du Z, Yang R, Guo Z, Song Y, Wang J. 2002. Identification of *Staphylococcus aureus* and determination of its methicillin resistance by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Anal Chem 74:5487–5491.

Dubois D, Leyssene D, Chacornac JP, Kostrzewa M, Schmit PO, Talon R, Bonnet R, Delmas J. 2010. Identification of a variety of *Staphylococcus* species by MALDI-TOF mass spectrometry. J Clin Microbiol 48:941–945.

Dworzanski JP, Dickinson DN, Deshpande SV, Snyder AP, Eckenrode BA. 2010. Discrimination and phylogenomic classification of *Bacillus anthracis-cereus-thuringiensis* strains based on LC-MS/MS analysis of whole cell protein digests. Anal Chem 82:145–155.

Ecker DJ, Sampath R, Massire C, Blyn LB, Hall TA, Eshoo MW, Hofstadler SA. 2008. Ibis T5000: A universal biosensor approach for microbiology. Nat Rev Microbiol 6:553–558.

Edwards-Jones V, Claydon MA, Evanson DJ, Walker J, Fox AJ, Gordon DB. 2000. Rapid discrimination between methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* by intact cell mass spectrometry. J Med Microbiol 49:295–300.

Elhanany E, Barak R, Fisher M, Kobiler D, Altboum Z. 2001. Detection of specific *Bacillus anthracis* spore biomarkers by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 15:2110–2116.

Eng JK, McCormack AL, Yates JR III. 1994. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. J Am Soc Mass Spectrom 5:976–989.

English RD, Warscheid B, Fenselau C, Cotter RJ. 2003. *Bacillus* spore identification via proteolytic peptide mapping with a miniaturized MALDI TOF mass spectrometer. Anal Chem 75:6886–6893.

Erhard M, von Dohren H, Jungblut P. 1997. Rapid typing and elucidation of new secondary metabolites of intact cyanobacteria using MALDI-TOF mass spectrometry. Nat Biotechnol 15:906–909.

Everley RA, Mott TM, Wyatt SA, Toney DM, Croley TR. 2008. Liquid chromatography/mass spectrometry characterization of *Escherichia coli* and *Shigella* species. J Am Soc Mass Spectrom 19:1621–1628.

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

Fagerquist CK, Miller WG, Harden LA, Bates AH, Vensel WH, Wang G, Mandrell RE. 2005. Genomic and proteomic identification of a DNA-binding protein used in the ‘‘fingerprinting’’ of *Campylobacter* species and strains by MALDI-TOF-MS protein biomarker analysis. Anal Chem 77:4897–4907.

Fagerquist CK, Bates AH, Heath S, King BC, Garbus BR, Harden LA, Miller WG. 2006. Sub-speciating *Campylobacter jejuni* by proteomic analysis of its protein biomarkers and their post-translational modifications. J Proteome Res 5:2527–2538.

Fagerquist CK, Garbus BR, Miller WG, Williams KE, Yee E, Bates AH, Boyle S, Harden LA, Cooley MB, Mandrell RE. 2010. Rapid identification of protein biomarkers of *Escherichia coli* O157: H7 by matrix-assisted laser desorption ionization-time-of-flight-time-of-flight mass spectrometry and top-down proteomics. Anal Chem 82:2717–2725.

Fenselau C, Demirev P. 2011. Rapid characterization of microorganisms by mass spectrometry: An overview. In: Fenselau C, Demirev P, editors. Rapid characterization of microorganisms by mass spectrometry. Washington: ACS. pp. 1–4.

Fenselau C, Russell S, Swatkoski S, Edwards N. 2007. Proteomic strategies for rapid characterization of micro-organisms. Eur J Mass Spectrom 13:35.

Fox K, Fox A, Rose J, Walla M. 2011. Speciation of coagulase negative staphylococci, isolated from indoor air, using SDS PAGE gel bands of expressed proteins followed by MALDI TOF MS and MALDI TOF-TOF MS-MS analysis of tryptic peptides. J Microbiol Methods 84:243–250.

Freiwald A, Sauer S. 2009. Phylogenetic classification and identification of bacteria by mass spectrometry. Nat Protocols 4:732–742.

Fujinami Y, Kikkawa HS, Kurosaki Y, Sakurada K, Yoshino M, Yasuda J. 2011. Rapid discrimination of *Legionella* by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Microbiol Res 166:77–86.

Gaia V, Casati S, Tonolla M. 2011. Rapid identification of *Legionella* spp. by MALDI-TOF MS based protein mass fingerprinting. Syst Appl Microbiol 34:40–44.

Gao B, Gupta R. 2012. Microbial systematics in the post-genomics era. Antonie van Leeuwenhoek 101:45–54.

Ghyselinck J, Van Hoorde K, Hoste B, Heylen K, De Vos P. 2011. Evaluation of MALDI-TOF MS as a tool for high-throughput dereplication. J Microbiol Methods 86:327–336.

Giebel RA, Fredenberg W, Sandrin TR. 2008. Characterization of environmental isolates of *Enterococcus* spp. by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Water Res 42:931–940.

Giebel R, Worden C, Rust SM, Kleinheinz GT, Robbins M, Sandrin TR. 2010. Microbial fingerprinting using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS): Applications and challenges. In: Laskin A, Sariaslani S, Gadd G, editors. Adv Appl Microbiol 71. San Diego: Elsevier. pp. 149–184.

Grosse-Herrenthey A, Maier T, Gessler F, Schaumann R, Böhnel H, Kostrzewa M, Krüger M. 2008. Challenging the problem of clostridial identification with matrix-assisted laser desorption and ionization-time-of-flight mass spectrometry (MALDI-TOF MS). Anaerobe 14:242–249.

Haag AM, Taylor SN, Johnston KH, Cole RB. 1998. Rapid identification and speciation of *Haemophilus* bacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J Mass Spectrom 33:750–756.

Hahn D, Mirza B, Benagli C, Vogel G, Tonolla M. 2011. Typing of nitrogen-fixing *Frankia* strains by matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry. Syst Appl Microbiol 34:63–68.

Hettick JM, Kashon ML, Slaven JE, Ma Y, Simpson JP, Siegel PD, Mazurek GN, Weissman DN. 2006. Discrimination of intact mycobacteria at the strain level: A combined MALDI-TOF MS and biostatistical analysis. Proteomics 6:6416–6425.

Holland RD, Wilkes JG, Rafii F, Sutherland JB, Persons CC, Voorhees KJ, Lay JO, Jr. 1996. Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 10:1227–1232.

Honisch C, Raghunathan A, Cantor CR, Palsson BØ, van den Boom D. 2004. High-throughput mutation detection underlying adaptive evolution of *Escherichia coli*-K12. Genome Res 14:2495–2502.

Honisch C, Chen Y, Mortimer C, Arnold C, Schmidt O, van den Boom D, Cantor CR, Shah HN, Gharbia SE. 2007. Automated comparative sequence analysis by base-specific cleavage and mass spectrometry for nucleic acid-based microbial typing. Proc Natl Acad Sci USA 104:10649–10654.

Horneffer V, Haverkamp J, Janssen HG, Notz R. 2004. MALDI-TOF-MS analysis of bacterial spores: Wet heat-treatment as a new releasing technique for biomarkers and the influence of different experimental parameters and microbiological handling. J Am Soc Mass Spectrom 15:1444–1454.

Hornik K, Leisch F. 2005. R version 2.1.0. Comput Stat 20:197–202.

Hotta Y, Teramoto K, Sato H, Yoshikawa H, Hosoda A, Tamura H. 2010. Classification of genus *Pseudomonas* by MALDI-TOF MS based on

ribosomal protein coding in *S10-spc-alpha* operon at strain level. J Proteome Res 9:6722–6728.

Hujer KM, Hujer AM, Endimiani A, Thomson JM, Adams MD, Goglin K, Rather PN, Pennella TD, Massire C, Eshoo MW, Sampath R, Blyn LB, Ecker DJ, Bonomo RA. 2009. Rapid determination of quinolone resistance in *Acinetobacter* spp. J Clin Microbiol 47:1436–1442.

Hurst G, Weaver K, Doktycz M, Buchanan M, Costello A, Lidstrom M. 1998. MALDI-TOF analysis of polymerase chain reaction products from methanotrophic bacteria RID A-7499-2011. Anal Chem 70: 2693–2698.

Hutchens TW, Yip TT. 1993. New desorption strategies for the mass-spectrometric analysis of macromolecules. Rapid Commun Mass Spectrom 7:576–580.

Ilina EN, Borovskaya AD, Malakhova MM, Vereshchagin VA, Kubanova AA, Kruglov AN, Svistunova TS, Gazarian AO, Maier T, Kostrzewa M, Govorun VM. 2009. Direct bacterial profiling by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry for identification of pathogenic *Neisseria*. J Mol Diagn 11:75–86.

Ilina EN, Borovskaya AD, Serebryakova MV, Chelysheva VV, Momynaliev KT, Maier T, Kostrzewa M, Govorun VM. 2010. Application of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for the study of *Helicobacter pylori*. Rapid Commun Mass Spectrom 24:328–334.

Jabbour R. 2011. Mass spectrometry for microbial proteomics. Anal Bioanal Chem 400:1815–1816.

Jabbour RE, Wade MM, Deshpande SV, Stanford MF, Wick CH, Zulich AW, Snyder AP. 2010. Identification of *Yersinia pestis* and *Escherichia coli* strains by whole cell and outer membrane protein extracts with mass spectrometry-based proteomics. J Proteome Res 9:3647–3655.

Jackson KA, Edwards-Jones V, Sutton CW, Fox AJ. 2005. Optimisation of intact cell MALDI method for fingerprinting of methicillin-resistant *Staphylococcus aureus*. J Microbiol Methods 62:273–284.

Jackson G, Mcnichols R, Fox G, Willson R. 2007. Universal bacterial identification by mass spectrometry of 16S ribosomal RNA cleavage products. Int J Mass Spectrom 261:218–226.

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

Karger A, Ziller M, Bettin B, Mintel B, Schares S, Geue L. 2010. Determination of serotypes of shiga toxin-producing *Escherichia coli* isolates by intact cell matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 77:896–905.

Kemptner J, Marchetti-Deschmann M, Mach R, Druzhinina IS, Kubicek CP, Allmaier G. 2009. Evaluation of matrix-assisted laser desorption/ionization (MALDI) preparation techniques for surface characterization of intact *Fusarium* spores by MALDI linear time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 23:877–884.

Keys CJ, Dare DJ, Sutton H, Wells G, Lunt M, McKenna T, McDowall M, Shah HN. 2004. Compilation of a MALDI-TOF mass spectral database for the rapid screening and characterisation of bacteria implicated in human infectious diseases. Infect Genet Evol 4:221–242.

Killgore G, Thompson A, Johnson S, Brazier J, Kuijper E, Pepin J, Frost EH, Savelkoul P, Nicholson B, van den Berg RJ, Kato H, Sambol SP, Zukowski W, Woods C, Limbago B, Gerding DN, McDonald LC. 2008. Comparison of seven techniques for typing international epidemic strains of *Clostridium difficile*: Restriction endonuclease analysis, pulsed-field gel electrophoresis, PCR-ribotyping, multilocus sequence typing, multilocus variable-number tandem-repeat analysis, amplified fragment length polymorphism, and surface layer protein A gene sequence typing. J Clin Microbiol 46:431–437.

Krader P, Emerson D. 2004. Identification of archaea and some extremophilic bacteria using matrix-assisted laster desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Extremophiles 8:259–268.

Kraushaar B, Dieckmann R, Wittwer M, Knabner D, Konietzny A, Mäde D, Strauch E. 2011. Characterization of a *Yersinia enterocolitica* biotype 1A strain harbouring an ail gene. J Appl Microbiol 111:997–1005.

Krishnamurthy T, Rajamani U, Ross PL. 1996. Detection of pathogenic and non-pathogenic bacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 10:883–888.

Krishnamurthy T, Deshpande S, Hewel J, Liu H, Wick C, Yatesiii J. 2007. Specific identification of *Bacillus anthracis* strains. Int J Mass Spectrom 259:140–146.

Kuehl B, Marten SM, Bischoff Y, Brenner-Weiß G, Obst U. 2011. MALDI-ToF mass spectrometry—Multivariate data analysis as a tool for classification of reactivation and non-culturable states of bacteria. Anal Bioanal Chem 401:1597–1604.

Lartigue MF, Héry-Arnaud G, Haguenoer E, Domelier AS, Schmit P-O, van der Mee-Marquet N, Lanotte P, Mereghetti L, Kostrzewa M, Quentin R. 2009. Identification of *Streptococcus agalactiae* isolates from various phylogenetic lineages by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 47:2284–2287.

Lasch P, Drevinek M, Nattermann H, Grunow R, Stämmler M, Dieckmann R, Schwecke T, Naumann D. 2010. Characterization of *Yersinia* using MALDI-TOF mass spectrometry and chemometrics. Anal Chem 82:8464–8475.

Leenders F, Stein TH, Kablitz B, Franke P, Vater J. 1999. Rapid typing of *Bacillus subtilis* strains by their secondary metabolites using matrix-assisted laser desorption/ionization mass spectrometry of intact cells. Rapid Commun Mass Spectrom 13:943–949.

Lefmann M, Honisch C, Böcker S, Storm N, von Wintzingerode F, Schlötelburg C, Moter A, van den Boom D, Göbel UB. 2004. Novel mass spectrometry-based tool for genotypic identification of mycobacteria. J Clin Microbiol 42:339–346.

Leuschner RGK, Beresford-Jones N, Robinson C. 2003. Difference and consensus of whole cell *Salmonella enterica* subsp. enterica serovars matrix-assisted laser desorption/ionization time-of-flight mass spectrometry spectra. Lett Appl Microbiol 38:24–31.

Liu H, Du Z, Wang J, Yang R. 2007. Universal sample preparation method for characterization of bacteria by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 73:1899–1907.

Lundquist M, Caspersen MB, Wikström P, Forsman M. 2005. Discrimination of *Francisella tularensis* subspecies using surface enhanced laser desorption ionization mass spectrometry and multivariate data analysis. FEMS Microbiol Lett 243:303–310.

Lynn EC, Chung M, Tsai W, Han C. 1999. Identification of *Enterobacteriaceae* bacteria by direct matrix-assisted laser desorptiom/ionization mass spectrometric analysis of whole cells. Rapid Commun Mass Spectrom 13:2022–2027.

Madigan MT, Martinko JM, Dunlap PV, Clark DP. 2009. Brock biology of microorganisms. CA: Pearson Education Inc. pp. 141–174.

Madonna AJ, Basile F, Furlong E, Voorhees KJ. 2001. Detection of bacteria from biological mixtures using immunomagnetic separation combined with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 15:1068–1074.

Madonna AJ, Cuyk SV, Voorhees KJ. 2003. Detection of *Escherichia coli* using immunomagnetic separation and bacteriophage amplification coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 17:257–263.

Majcherczyk PA, McKenna T, Moreillon P, Vaudaux P. 2006. The discriminatory power of MALDI-TOF mass spectrometry to differentiate between isogenic teicoplanin-susceptible and teicoplanin-resistant strains of methicillin-resistant *Staphylococcus aureus*. FEMS Microbiol Lett 255:233–239.

Mandrell RE, Harden LA, Bates A, Miller WG, Haddon WF, Fagerquist CK. 2005. Speciation of *Campylobacter coli*, *C. jejuni*, *C. helveticus*,

C. lari, *C. sputorum*, and *C. upsaliensis* by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 71:6292–6307.

Marklein G, Josten M, Klanke U, Müller E, Horré R, Maier T, Wenzel T, Kostrzewa M, Bierbaum G, Hoerauf A, Sahl HG. 2009. MALDI-TOF mass-spectrometry for fast and reliable identification of clinical yeast isolates. J Clin Microbiol 47:2912–2917.

Mellmann A, Cloud J, Maier T, Keckevoet U, Ramminger I, Iwen P, Dunn J, Hall G, Wilson D, LaSala P, Kostrzewa M, Harmsen D. 2008. Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry in comparison to 16S rRNA gene sequencing for species identification of nonfermenting bacteria. J Clin Microbiol 46:1946–1954.

Mellmann A, Bimet F, Bizet C, Borovskaya AD, Drake RR, Eigner U, Fahr AM, He Y, Ilina EN, Krostrzewa M, Maier T, Mancinelli L, Moussaoui W, Prévost G, Putignani L, Seachord CL, Tang YW, Harmsen D. 2009. High interlaboratory reproducibility of matrix-assisted laser desorption ionization-time of flight mass spectrometry-based species identification of nonfermenting bacteria. J Clin Microbiol 47:3732–3734.

Mortz E. 1996. Sequence tag identification of intact proteins by matching tandem mass spectral data against sequence databases. Proc Natl Acad Sci USA 93:8264–8267.

Mott TM, Everley RA, Wyatt SA, Toney DM, Croley TR. 2010. Comparison of MALDI-TOF/MS and LC-QTOF/MS methods for the identification of enteric bacteria. Int J Mass Spectrom 291:24–32.

Moura H, Woolfitt AR, Carvalho MG, Pavlopoulos A, Teixeira LM, Satten GA, Barr JR. 2008. MALDI-TOF mass spectrometry as a tool for differentiation of invasive and noninvasive *Streptococcus pyogenes* isolates. FEMS Immunol Med Microbiol 53:333–342.

Muddiman D, Wunschel D, Liu C, PasaTolic L, Fox K, Fox A, Anderson G, Smith RD. 1996. Characterization of PCR products from bacilli using electrospray ionization FTICR mass spectrometry RID F-3820-2010. Anal Chem 68:3705–3712.

Mukherjee AK, Das K. 2005. Correlation between diverse cyclic lipopeptides production and regulation of growth and substrate utilization by *Bacillus subtilis* strains in a particular habitat. FEMS Microbiol Ecol 54:479–489.

Mukhopadhya I, Hansen R, Nicholl CE, Alhaidan YA, Thomson JM, Berry SH, Pattinson C, Stead DA, Russell RK, El-Omar EM, Hold GL. 2011. A comprehensive evaluation of colonic mucosal isolates of *Sutterella wadsworthensis* from inflammatory bowel disease. PLoS ONE 6:e27076.

Munoz R, López-López A, Urdiain M, Moore ERB, Rosselló-Móra R. 2011. Evaluation of matrix-assisted laser desorption ionization-time of flight whole cell profiles for assessing the cultivable diversity of aerobic and moderately halophilic prokaryotes thriving in solar saltern sediments. Syst Appl Microbiol 34:69–75.

Murray PR. 2010. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry: Usefulness for taxonomy and epidemiology. Clin Microbiol Infect 16:1626–1630.

Nilsson CL. 1999. Fingerprinting of *Helicobacter pylori* strains by matrix-assisted laser desorption/ionization mass spectrometric analysis. Rapid Commun Mass Spectrom 13:1067–1071.

Ochoa ML, Harrington PB. 2005. Immunomagnetic isolation of enterohemorrhagic *Escherichia coli* O157: H7 from ground beef and identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and database searches. Anal Chem 77:5258–5267.

Parolo CC, Do T, Henssqe U, Alves LS, de Santan Giongo FE, Corcao G, Maltz M, Beighton D. 2011. Genetic diversity of *Lactobacillus paracasei* isolated from in situ human oral biofilms. J Appl Microbiol 111:105–113.

Pennanec X, Dufour A, Haras D, Réhel K. 2010. A quick and easy method to identify bacteria by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 24:384–392.

Perera MR, Vanstone VA, Jones MGK. 2005. A novel approach to identify plant parasitic nematodes using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 19:1454–1460.

Pierce CY, Barr JR, Woolfitt AR, Moura H, Shaw EI, Thompson HA, Massung RF, Fernandez FM. 2007. Strain and phase identification of the U.S. category B agent *Coxiella burnetii* by matrix assisted laser desorption/ionization time-of-flight mass spectrometry and multivariate pattern recognition. Anal Chim Acta 583:23–31.

Price NPJ, Rooney AP, Swezey JL, Perry E, Cohan FM. 2007. Mass spectrometric analysis of lipopeptides from *Bacillus* strains isolated from diverse geographical locations. FEMS Microbiol Lett 271: 83–89.

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

Rezzonico F, Vogel G, Duffy B, Tonolla M. 2010. Application of whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry for rapid identification and clustering analysis of *Pantoea* species. Appl Environ Microbiol 76:4497–4509.

Robbins M, Giebel R, Sandrin T, Kleinheinz GT. 2007. Matrix assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF-MS) analysis of Sunset Park *Escherichia coli* isolates. Great Lakes Beach Association/State of the Lake Annual Meeting, November 3–5, Traverse City, MI.

Rubbenstroth D, Hotzel H, Knobloch J, Teske L, Rautenschlein S, Ryll M. 2011. Isolation and characterization of atypical *Riemerella columbina* strains from pigeons and their differentiation from *Riemerella anatipestifer*. Vet Microbiol 147:103–112.

Ruelle V, Moualij BE, Zorzi W, Ledent P, Pauw ED. 2004. Rapid identification of environmental bacterial strains by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 18:2013–2019.

Rupf S, Breitung K, Schellenberger W, Merte K, Kneist S, Eschrich K. 2005. Differentiation of mutans streptococci by intact cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Oral Microbiol Immunol 20:267–273.

Ryzhov V, Fenselau C. 2001. Characterization of the protein subset desorbed by MALDI from whole bacterial cells. Anal Chem 73:746–750.

Ryzhov V, Hathout Y, Fenselau C. 2000. Rapid characterization of spores of *Bacillus cereus* group bacteria by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry. Appl Environ Microbiol 66:3828–3834.

Sato H, Teramoto K, Ishii Y, Watanabe K, Benno Y. 2011. Ribosomal protein profiling by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for phylogenety-based subspecies resolution of *Bifidobacterium longum*. Syst Appl Microbiol 34:76–80.

Sauer S, Kliem M. 2010. Mass spectrometry tools for the classification and identification of bacteria. Nature Rev Microbiol 8:74–82.

Sauer S, Freiwald A, Maier T, Kube M, Reinhardt R, Kostrzewa M, Geider K. 2008. Classification and identification of bacteria by mass spectrometry and computational analysis. PLoS ONE 3:e2843.

Schaller A, Troller R, Molina D, Gallati S, Aebi C, Stutzmann Meier P. 2006. Rapid typing of*Moraxella catarrhalis* subpopulations based on outer membrane proteins using mass spectrometry. Proteomics 6:172–180.

Schmid O, Ball G, Lancashire L, Culak R, Shah H. 2005. New approaches to identification of bacterial pathogens by surface enhanced laser desorption/ionization time of flight mass spectrometry in concert with artificial neural networks, with special reference to *Neisseria gonorrhoeae*. J Med Microbiol 54:1205–1211.

Schmidt F, Fiege T, Hustoft HK, Kneist S, Thiede B. 2009. Shotgun mass mapping of *Lactobacillus* species and subspecies from caries related isolates by MALDI-MS. Proteomics 9:1994–2003.

Schumaker S, Borror CM, Sandrin TR. 2012. Automating data acquisition affects mass spectrum quality and reproducibility during bacterial profiling using an intact cell sample preparation method with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 26:243–253.

Šedo O, Sedláček I, Zdráhal Z. 2011. Sample preparation methods for MALDI-MS profiling of bacteria. Mass Spectrom Rev 30:417–434.

Seibold E, Bogumil R, Vorderwülbecke S, Al Dahouk S, Buckendahl A, Tomaso H, Splettstoesser W. 2007. Optimized application of surface-enhanced laser desorption/ionization time-of-flight MS to differentiate *Francisella tularensis* at the level of subspecies and individual strains. FEMS Immunol Med Microbiol 49:364–373.

Seng P, Drancourt M, Gouriet F, La Scola B, Fournier P, Rolain JM, Raoult D. 2009. Ongoing revolution in bacteriology: Routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Infect Dis 49:543–551.

Shah HN, Rajakaruna L, Ball G, Misra R, Al-Shahib A, Fang M, Gharbia SE. 2011. Tracing the transition of methicillin resistance in sub-populations of *Staphylococcus aureus*, using SELDI-TOF mass spectrometry and artificial neural network analysis. Syst Appl Microbiol 34:81–86.

Siegrist TJ, Anderson PD, Huen WH, Kleinheinz GT, McDermott CM, Sandrin TR. 2007. Discrimination and characterization of environmental strains of *Escherichia coli* by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). J Microbiol Methods 68:554–562.

Stackebrandt E, Päuker O, Erhard M. 2005. Grouping *Myxococci* (*Corallococcus*) strains by matrix-assisted laser desorption ionization time-of-flight (MALDI TOF) mass spectrometry: Comparison with gene sequence phylogenies. Curr Microbiol 50:71–77.

Stephan R, Ziegler D, Pfluger V, Vogel G, Lehner A. 2010. Rapid genus- and species-specific identification of *Cronobacter* spp. by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 48:2846–2851.

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

Stump MJ, Black G, Fox A, Fox KF, Turick CE, Matthews M. 2005. Identification of marker proteins for *Bacillus anthracis* using MALDI-TOF MS and ion trap MS/MS after direct extraction or electrophoretic separation. J Sep Sci 28:1642–1647.

Sun L, Teramoto K, Sato H, Torimura M, Tao H, Shintani T. 2006. Characterization of ribosomal proteins as biomarkers for matrix-assisted laser desorption/ionization mass spectral identification of *Lactobacillus plantarum*. Rapid Commun Mass Spectrom 20:3789–3798.

Swaminathan B, Barrett T, Hunter S, Tauxe R. 2001. PulseNet: The molecular subtyping network for foodborne bacterial disease surveillance, United States. Emerging Infect Dis 7:382–389.

Syrmis MW, Moser RJ, Whiley DM, Vaska V, Coombs GW, Nissen MD, Sloots TP, Nimmo GR. 2011. Comparison of a multiplexed MassARRAY system with real-time allele-specific PCR technology for genotyping of methicillin-resistant *Staphylococcus aureus*. Clin Microbiol Infect 17:1804–1810.

Szabados F, Woloszyn J, Richter C, Kaase M, Gatermann S. 2010. Identification of molecularly defined *Staphylococcus aureus* strains using matrix-assisted laser desorption/ionization time of flight mass spectrometry and the Biotyper 2.0 database. J Med Microbiol 59:787–790.

Tang N, Tornatore P, Weinberger SR. 2004. Current developments in SELDI affinity technology. Mass Spectrom Rev 23:34–44.

Tanigawa K, Kawabata H, Watanabe K. 2010. Identification and typing of *Lactococcus lactis* by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 76:4055–4062.

Tenover FC, Arbeit RD, Goering RV. 1997. How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections: A review for healthcare epidemiologists. Infect Control Hosp Epidemiol 18:426–439.

Teramoto K, Sato H, Sun L, Torimura M, Tao H. 2007a. A simple intact protein analysis by MALDI-MS for characterization of ribosomal proteins of two genome-sequenced lactic acid bacteria and verification of their amino acid sequences. J Proteome Res 6:3899–3907.

Teramoto K, Sato H, Sun L, Torimura M, Tao H, Yoshikawa H, Hotta Y, Hosoda A, Tamura H. 2007b. Phylogenetic classification of *Pseudomonas putida* strains by MALDI-MS using ribosomal subunit proteins as biomarkers. Anal Chem 79:8712–8719.

Teramoto K, Kitagawa W, Sato H, Torimura M, Tamura T, Tao H. 2009. Phylogenetic analysis of *Rhodococcus erythropolis* based on the variation of ribosomal proteins as observed by matrix-assisted laser desorption ionization-mass spectrometry without using genome information. J Biosci Bioeng 108:348–353.

Valentine NB, Wahl JH, Kingsley MT, Wahl KL. 2002. Direct surface analysis of fungal species by matrix-assisted laser desorption/ionization mass spectrometry. Rapid Commun Mass Spectrom 16:1352–1357.

van Veen SQ, Claas ECJ, Kuijper EJ. 2010. High-throughput identification of bacteria and yeast by matrix-assisted laser desorption ionization-time of flight mass spectrometry in conventional medical microbiology laboratories. J Clin Microbiol 48:900–907.

Vanlaere E, Sergeant K, Dawyndt P, Kallow W, Erhard M, Sutton H, Dare D, Devreese B, Samyn B, Vandamme P. 2008. Matrix-assisted laser desorption ionisation-time-of-flight mass spectrometry of intact cells allows rapid identification of *Burkholderia cepacia* complex. J Microbiol Methods 75:279–286.

Vargha M, Takáts Z, Konopka A, Nakatsu CH. 2006. Optimization of MALDI-TOF MS for strain level differentiation of *Arthrobacter* isolates. J Microbiol Methods 66:399–409.

von Wintzingerode F, Böcker S, Schlötelburg C, Chiu NHL, Storm N, Jurinke C, Cantor CR, Göbel UB, van den Boom D. 2002. Base-specific fragmentation of amplified 16S rRNA genes analyzed by mass spectrometry: A tool for rapid bacterial identification. Proc Natl Acad Sci USA 99:7039–7044.

Vorob'eva LI, Khasaeva FM, Vasilyuk NV, Trenquil E. 2011. Characterization of propionic acid bacteria using matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. Microbiology 80:664–671.

Walker J, Fox AJ, Edwards-Jones V, Gordon DB. 2002. Intact cell mass spectrometry (ICMS) used to type methicillin-resistant *Staphylococcus aureus*: Media effects and inter-laboratory reproducibility. J Microbiol Methods 48:117–126.

Welham KJ, Domin MA, Scannell DE, Cohen E, Ashton DS. 1998. The characterization of micro-organisms by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 12:176–180.

Welker M. 2011. Proteomics for routine identification of microorganisms. Proteomics 11:3143–3153.

Wensing A, Zimmermann S, Geider K. 2010. Identification of the corn pathogen *Pantoea stewartii* by mass spectrometry of whole-cell extracts and its detection with novel PCR primers. Appl Environ Microbiol 76:6248–6256.

Wiesner A. 2004. Detection of tumor markers with ProteinChip technology. Curr Pharm Biotechnol 5:45–67.

Wilkes JG, Tucker RK, Montgomery JA, Cooper WM, Sutherland JB, Buzatu DA. 2012. Reduction of food matrix interference by a combination of sample preparation and multi-dimensional gating techniques to facilitate rapid, high sensitivity analysis for *Escherichia coli* serotype O157 by flow cytometry. Food Microbiol 30:281–288.

Williams TL, Andrzejewski D, Lay JO, Musser SM. 2003. Experimental factors affecting the quality and reproducibility of MALDI TOF mass spectra obtained from whole bacteria cells. J Am Soc Mass Spectrom 14:342–351.

Williamson YM, Moura H, Woolfitt AR, Pirkle JL, Barr JR, Carvalho MDG, Ades EP, Carlone GM, Sampson JS. 2008. Differentiation of *Streptococcus pneumoniae* conjunctivitis outbreak isolates by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 74:5891–5897.

Wolters M, Rohde H, Maier T, Belmar-Campos C, Franke G, Scherpe S, Aepfelbacher M, Christner M. 2011. MALDI-TOF MS fingerprinting allows for discrimination of major methicillin-resistant *Staphylococcus aureus* lineages. Int J Med Microbiol 301: 64–68.

Wynne C, Fenselau C, Demirev PA, Edwards N. 2009. Top-down identification of protein biomarkers in bacteria with unsequenced genomes. Anal Chem 81:9633–9642.

Wynne C, Edwards NJ, Fenselau C. 2010. Phyloproteomic classification of unsequenced organisms by top-down identification of bacterial proteins using capLC-MS/MS on an Orbitrap. Proteomics 10:3631–3643.

Zhang JI, Costa AB, Tao WA, Cooks RG. 2011. Direct detection of fatty acid ethyl esters using low temperature plasma (LTP) ambient ionization mass spectrometry for rapid bacterial differentiation. Analyst 136:3091–3097.