Chapter 11
Rapid Profiling of Human Pathogenic Bacteria and Antibiotic Resistance Employing Specific Tryptic Peptides as Biomarkers

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Introduction

Identification of human pathogens by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry, based on profiling of mainly taxonomic relevant ribosomal proteins and comparison to a reference mass spectra database, has developed into a robust cutting-edge diagnostic technology and has *revolutionized work in microbiological laboratories in recent years* (Seng et al. 2009). *This is due to the* high speed of analysis allowing a short time to result and the streamlined protocol, enabling an accurate and cost-effective identification within less than 20 min. *Application fields include* clinical and veterinary diagnostics, food safety control, outbreak tracking, environmental microbiology, biotechnology, and biodefense. A major challenge to MS-based identification has been to reliably increase the taxonomic resolution to the below-species level. This challenge originates from the fact that strains of one species exhibit substantial genetic overlap and thus high protein similarity. Two main approaches have been applied to resolve mass peak variations below species level: the library-based and the proteomics-based approach. Within the library-based approach, both sample pre-treatment and data reduction strategies have been developed. Proteomics-based approaches comprise bottom-up and top-down characterization of biomarkers applying large databases available to the public.

The focus of this chapter is on the state of MALDI-TOF- and MALDI-TOF/TOF MS-based identification of human pathogens below the species level and specifically on the application of tryptic peptides as a recent development in enhancing the

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discriminatory power for bacterial profiling and determination of bacterial antibiotic resistances. Rapid identification at the below-species level is highly important in identification and diagnosis of pathogens, to determine appropriate drug therapy, to reliably trace back contamination sources in elucidation of epidemics, to improve food production and processing, or to develop better clinical practices.

Library-Based Approaches

In library-based approaches, peak lists extracted from a profile mass spectrum of unknown microorganisms are compared to the peak lists of reference spectra deposited in a database containing a large collection of well-characterized strains. This approach based on the detection of subtle and reproducible differences in spectra has been applied in most studies reporting successful profiling of pathogens below the species level using MALDI-TOF MS (Table 11.1). A comprehensive review on MALDI-TOF MS profiling of bacteria at the strain level has recently been published by Sandrin et al. (2013). The prerequisite for MALDI-TOF MS profiling is cultivation of pathogens on solid or liquid culture media and subsequently direct smearing of inactivated whole cells onto the MALDI target or short chemical extraction with formic acid and acetonitrile and spotting of supernatants onto the MALDI target. In general, the number of proteins detected increases with the level of separation and fractions collected which helps to increase taxonomic resolution. In both cases (direct smear or extract), the sample is covered with a standard MALDI matrix, for example, α-cyano-4-hydroxycinnamic acid. Mass spectra are acquired in positive ion mode from random locations on the target spot and comprise a mass range of 2–20 kDa (Ghyselinck et al. 2011; Ilina et al. 2010), of a broader (Hettick et al. 2006; Jackson et al. 2005; Teramoto et al. 2009) or narrower mass range (Keys et al. 2004; Rajakaruna et al. 2009). In particular, ions with high masses are promising for differentiation in below-species level due to rarity of these ions and to the absence of background signals in that mass range. Single mass peaks of spectra in library-based approaches are not given proof of identity; however, most of the peaks are attributed to basic, abundant, and conserved proteins, in particular ribosomal proteins (Sauer and Kliem 2010; Fenselau and Demirev 2001) and to a certain degree to proteins associated with bacterial cell walls (Evason et al. 2001). Ribosomal proteins comprise approximately 30 % of total proteins in a cell being in the exponential growth phase. Success in identification below the species level using library-based approaches requires robust software, reliable algorithms as well as databases in order to precisely compare acquired spectra to database entries and to calculate the similarity. Furthermore, mass spectral quality (resolution, accuracy, and reproducible acquisition of spectra) is key, and standardized experimental conditions including culture conditions need to be strictly followed in order to ensure reproducibility of the MALDI mass spectra and to detect specific protein biomarker masses for organisms below the species level.

Table 11.1 Overview of studies for profiling human pathogens with MALDI-TOF MS at the strain level: Examples of strain categorization (A), strain differentiation (B), and strain identification (C). *MRSA* methicillin-resistant *Staphylococcus aureus, MSSA* methicillin-sensitive *S. aureus, L* library-based approach, *P* proteomics-based approach, *CHCA* a-cyano-4-hydroxycinnamic acid, *CMBT* 5-chloro-2-mercaptobenzothiazole, *DHB* 2-hydroxy-5-methoxy benzoic acid, *FA* ferulic acid, *HABA* 2,4-hydroxyphenylazobenzoic acid, *SA* 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), *THAP* 2,4,6-trihydroxyacetophenon. Adapted and modified from Sandrin et al. (2013)

Bacterium	Approach	Cell preparation	Matrix	Software	Instrument	Reference
A						
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 et al. 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	P	I/E	CHCA	MASCOT	Ultraflex; Bruker Saxonia Analytik	Schaller et al. 2006
Escherichia coli	L	I	SA	Diversity Database; Bio-Rad	Reflex IV; Bruker Daltonics	Siegrist et al. 2007
Escherichia coli	P	E	SA	ns	Voyager-DE STR; Applied Biosystems	Camara and Hays 2007
Enterococcus	L	E	SA	GelCompar II; Applied Maths	Reflex IV; Bruker Daltonics	Giebel et al. 2008

Table 11.1 (continued)

Bacterium	Approach	Cell preparation	Matrix	Software	Instrument	Reference
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
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
Staphylococcus	L	E	CHCA		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. 2011
MRSA	L	E	CHCA	BioTyper; Bruker Daltonics	Microflex LT; Bruker Daltonics	Wolters et al. 2011
MRSA	L	E	SA	ns	PBS II Protein Chip Array Reader; Ciphergen Biosystems	Shah et al. 2011
Yersinia enterocolitica	L, P	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						
Gram (−)/Gram (+) enterobacteria	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 et al. 1996

Table 11.1 (continued)

Bacterium	Approach	Cell preparation	Matrix	Software	Instrument	Reference
Cyanobacteria	L	I	CHCA	ns	Voyager Elite; PerSeptive Biosystems	Erhard et al. 1997
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 et al. 2000
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; Kratos Analytica	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 2E; 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	Reflex II; Bruker Daltonics	Fagerquist et al. 2005
Campylobacter	L	E	FA	ns	Reflex II; Bruker Daltonics	Mandrell et al. 2005
Escherichia coli	P	I	CHCA, SA, FA	Masslynx; Waters Corp.	MALDI LR; Micromass Ltd.	Ochoa and Harrington 2005
Aeromonas	L	I	SA	ns	BiFlex III; Bruker Daltonics	Donohue et al. 2006
Campylobacter	P	E	FA	in-house	Reflex II; Bruker Daltonics	Fagerquist et al. 2006

Table 11.1 (continued)

Bacterium	Approach	Cell preparation	Matrix	Software	Instrument	Reference
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
Bacillus anthracis, Bacillus cereus	P	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,P	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
Escherichia coli	L	I	SA	MATLAB; Math Works	MALDI LR; Micromass Ltd.	Chen et al. 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	P	E	CHCA, SA	In-house	4800 TOF-TOF; Applied Biosystems	Fagerquist et al. 2010
Pseudomonas	P	I	SA	ns	Axima Performance; Shimadzu/Kratos	Hotta et al. 2010
Enterobacteriaceae	L	E	SA	ns	Ultraflex II; Bruker Daltonics	Mott et al. 2010
Legionella	L	E	CHCA	BioTyper, Bruker Daltonics	Autoflex II; Bruker Daltonics	Fujinami et al. 2011

Table 11.1 (continued)

Bacterium	Approach	Cell preparation	Matrix	Software	Instrument	Reference
C						
Bordetella, as-yet-uncultured	P	E	3-HPA	SEQUENOM; Sequenom		Von Wintzingerode et al. 2002
Micrococcaceae (CoNS)	L	I	DHB	BGP database	Autoflex; Bruker Daltonics	Carbonnelle et al. 2007
Neisseria meningitidis	P	E	3-HPA	SEQUENOM; Sequenom	ns	Honisch et al. 2007
Stenotrophomonas maltophilia	L	E	3-HPA & DAC	Data Explorer; Applied Biosystems		Jackson et al. 2007
Salmonella	P	I	SA, CHCA, DHB	SARAMIS; bioMérieux	Ultraflex II TOF-TOF; Bruker Daltonics	Dieckmann et al. 2008
Streptococcus agalactiae	L	E	CHCA	BioTyper; Bruker Daltonics	Ultraflex III TOF-TOF; Bruker Daltonics	Lartigue et al. 2009
MRSA	P	E	3-HPA	MassARRAY Typer; Sequenom	MassArray Compact Analyzer; Sequenom	Syrmis et al. 2011

E extract; *I* intact; *ns* not specified

Regarding strain categorization, serovars of *Salmonella enterica* subsp. *enterica* have been successfully categorized by comparison of their MALDI mass spectra, which contained up to 500 mass peaks in that study (Leuschner et al. 2003). Karger et al. (2011) employed a library-based approach to categorize STEC serovars (Karger et al. 2011), and Stephan et al. (2011) categorized *Yersinia enterocolitica* as pathogenic or non-pathogenic strains (Stephan et al. 2011). Further studies showed that strains of *Yersinia pestis* could be categorized according to their biotypes (Ayyadurai et al. 2010), strains of *Escherichia coli* according to their environmental origin, and clinical strains of *Moraxella catarrhalis* have been categorized at the subpopulation level (Schaller et al. 2006). *Listeria monocytogenes* was categorized at the level of clonal lineage, whereby the MALDI MS-derived lineage agreed with those from pulsed-field gel electrophoresis (Barbuddhe et al. 2008). Categorization of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *Staphylococcus aureus* (MSSA) strains using characteristic markers for the methicillin resistance status has been achieved by Edwards-Jones et al. (2000) and by Shah et al. (2011) using artificial neural networks. Wolters et al. (2011) and recently, Josten et al. (2013) categorized strains according to the major clonal complexes of MRSA.

In order to differentiate between single bacterial strains, MALDI-TOF mass spectra have been used to identify mass peaks as biomarkers for the respective strains. Such an approach has been applied to *Helicobacter pylori* (Nilsson 1999), *E. coli* (Lynn et al. 1999), *Campylobacter* (Mandrell et al. 2005), *Mycobacterium* (Hettick et al. 2006), and MRSA (Majcherczyk et al. 2006). Williamson et al. (2008) differentiated strains of *Streptococcus pneumoniae* by using unique mass peaks (Williamson et al. 2008). Masses in the range of 5000–11,000 Da matched ribosomal proteins of *S. pneumoniae,* and spectrum clustering revealed the relationship between an outbreak of *S. pneumoniae* conjunctivitis and their corresponding isolates. Similarly, *Streptococcus pyogenes* strains could be differentiated by Moura et al. (2008) into invasive and non-invasive isolates using specific biomarkers (Moura et al. 2008). Many of the biomarker masses in the range of 4000–14,000 Da matched *S. pyogenes* ribosomal proteins. Differentiation of *Enterococcus faecium* and *E. faecalis* at the strain level has been described by Albesharat et al. (2011). Intact mycobacteria could be differentiated at the strain level by linear discriminant analysis (Hettick et al. 2006). Differentiation has been successful even when the intensity of the mass peaks was considered additionally to the presence or absence data. Pierce et al. (2007) demonstrated differentiation of *Coxiella burnetii* strains using partial least squares discriminant analysis of MALDI-TOF mass spectral peaks (Pierce et al. 2007).

For identification of single, unknown strains—compared to categorization or differentiation—the entire mass spectrum is usually used and compared to a library of reference spectra of known strains. Distinct algorithms have been applied in correlations calculated and often small spectral differences between strains that have been given more weight (weighted pattern matching) increased the sensitivity of such small differences and thus contributed to successful identification, for example, in studies using *E. coli* (Arnold and Reilly 1998), or Micrococcaceae (Carbonnelle

et al. 2007). In the study by Arnold and Reilly (1998), strains exhibited both peaks in common and also strain-specific peaks in the range of 3.5–10 kDa. By applying an algorithm calculating both cross-correlation and auto-correlation values for each of 13 intervals, 25 strains could be distinguished. Bright et al. (2002) applied a pattern recognition algorithm to the mass spectra (m/z 500–10,000), and each spectrum was translated into a point vector in an n-dimensional space. Data of 35 strains from 20 species and mainly enterobacteria were included in a reference library and correct identification on the strain level was achieved for 79% of the samples. The algorithm succeeded even in the distinction of species for which biochemical typing fails, for example, for *E. coli* O122 and *Citrobacter freundii*. A hierarchical cluster algorithm combined with analysis of variance (ANOVA) was used in a study by Hsieh et al. to extract biomarkers from several isolates of six human pathogens (Hsieh et al. 2008).

In general, two kinds of algorithms exist: one includes intensities of peaks and the other uses the presence and absence of mass peaks. It is worth mentioning that spectral mass signals exhibit an analytical error due to slight variability of acceleration voltage, to status of matrix crystals, and to peak recognition by the software. With respect to linear MALDI-TOF MS, an analytical error of approximately 500 ppm, meaning a 5 Da deviation for a signal at $m/z = 10{,}000$, is generally regarded acceptable. Besides software applications developed in-house, two main commercially available and automated softwares including validated reference databases are available (BioTyper, Bruker Daltonics (Sauer et al. 2008) and SARAMIS, bioMérieux (Kallow et al. 2000)), which also allow analysis of MALDI-TOF mass spectra on the below-species level as shown, for example, by Grosse-Herrenthey et al. (2008) using BioTyper to identify clostridia at the strain level or by Stephan et al. (2011) using SARAMIS for characterization of *Y. enterocolitica* strains according to their biotype. Such databases are constantly improving by inclusion of new bacteria relevant to clinical diagnostics, veterinary medicine, food safety, and environmental microbiology. To obtain more mass peaks serving as putative biomarkers and to increase sensitivity, in several studies samples have been treated by enzymes, detergent, sonication, corona plasma discharge, or heat (Nilsson 1999; Horneffer et al. 2004; Krishnamurthy et al. 1996; Ryzhov et al. 2000). Furthermore, in some studies mass spectra that contained less peaks have been applied for discrimination of strains as shown, for example for *M. catarrhalis* strains (Schaller et al. 2006), *S. aureus* (Shah et al. 2011), or *Francisella tularensis* (Seibold et al. 2007). In the latter study, a method applying surface-enhanced laser desorption/ionization has been used.

Proteomics-Based Approaches

The rapid increase in the availability of full genomes of bacteria in public databases boosted research of proteomics-based approaches comprising identification of single peaks in mass spectra in order to profile pathogens below the species level. Both

application of MALDI-TOF MS and MALDI-TOF/TOF MS have been described for identification of intact proteins serving as biomarkers. This comprises the use of their masses which are compared to in silico-generated protein databases derived from genomic databases. Intact protein identification has been successfully used to identify strain-specific protein biomarkers, for example, for *E. coli* O157:H7 (Ochoa and Harrington 2005), *Campylobacter* (Mandrell et al. 2005), and *Salmonella* (Dieckmann et al. 2008).

In bottom-up approaches, proteins extracted from bacterial cultures are digested enzymatically at specific sites and resulting peptides are identified by MS/MS (post-source decay, laser-induced dissociation, or collision-induced dissociation). Site-specific digestion is generally performed using proteolytic enzymes such as trypsin (Aebersold and Mann 2003; Yao et al. 2002). In order to accelerate digestion, microwave heating has been successfully applied (Lill et al. 2007). Non-enzymatic protein digestion by acid hydrolysis accelerated through microwave heating has been performed for analyzing spores of *Bacillus* (Swatkoski et al. 2006). Bottom-up approaches often include a separation and purification step prior to digestion. Fagerquist et al. (2005) applied high-performance liquid chromatography (HPLC) and 1D sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to proteins from *Campylobacter* before identifying strain-specific biomarker proteins. Two-dimensional SDS-PAGE has been used by Schaller et al. (2006) prior to biomarker identification for *M. catarrhalis* strains.

Regarding further optimization and speeding-up of bottom-up identification workflows, a proteomics-based approach that was developed recently to identify subspecies of *Salmonella enterica* (Gekenidis et al. 2014) is described in detail in the next chapter. This approach comprises whole-cell protein extracts produced via an established extraction procedure (MALDI biotyping) and high-intensity focused ultrasound (HIFU)-assisted trypsin digestion prior to identification of specific peptides and proteins.

In contrast, top-down proteomics approaches in profiling bacteria comprise accurate measurement of the mass of intact proteins and fragmentation of these by MS/MS yielding partial amino acid sequences and/or peptide fragments. Fragmentation is achieved by collision-induced dissociation, laser-induced dissociation, electron capture dissociation, or electron transfer dissociation. Resulting MS/MS spectra are compared to a database in order to identify the protein and ultimately—in the case of sufficiently unique protein sequence—the source strain. Software applications compare the masses of MS/MS fragment ions to a database of in silico fragment ions (a-, b-, and y-fragment ions) derived from a large number of protein sequences which exhibit the same mass as that of the biomarker. An algorithm calculates the probability of identification. MALDI-TOF/TOF MS has been used for identification of intact spores that were treated with 10 % formic acid on-target to facilitate extraction of small acid soluble proteins (Demirev et al. 2005). In another study, proteins were extracted with water–acetonitrile–TFA under bead-beating using 0.1 mm zirconia/silica beads for 1 min prior to biomarker identification of *E. coli* O157:H7 via MALDI-TOF/TOF MS (Fagerquist et al. 2010). Furthermore, shiga toxins of *E. coli* O157:H7 have been identified by this approach (Fagerquist and Sultan 2011) .

Future applications of MALDI in top-down approaches will need further developments to make fragmentation of large proteins more efficient (McLuckey 2010). Compared to library-based approaches, proteomics-based approaches are at an advantage with higher level of specificity and independence of producing mass spectral profiles with reproducible relative intensities of mass peaks.

Approaches Based on Tryptic Peptides Toward Identification and Typing of Pathogens Below the Species Level

Exploiting MALDI-TOF/TOF MS for Discrimination of Subspecies: In Search of Microorganism-Specific Tryptic Peptides

One approach to increase the taxonomic resolution of classical MALDI-TOF biotyping is by analyzing protein digests in the so-called bottom-up approach (as reviewed above). We have recently described a method for discrimination of bacterial subspecies relying on the ultra-fast generation of tryptic peptides enabling the identification of subspecies-specific biomarker peptides (Fig. 11.1). For the proof of concept, we used a model system consisting of the three *Salmonella enterica*

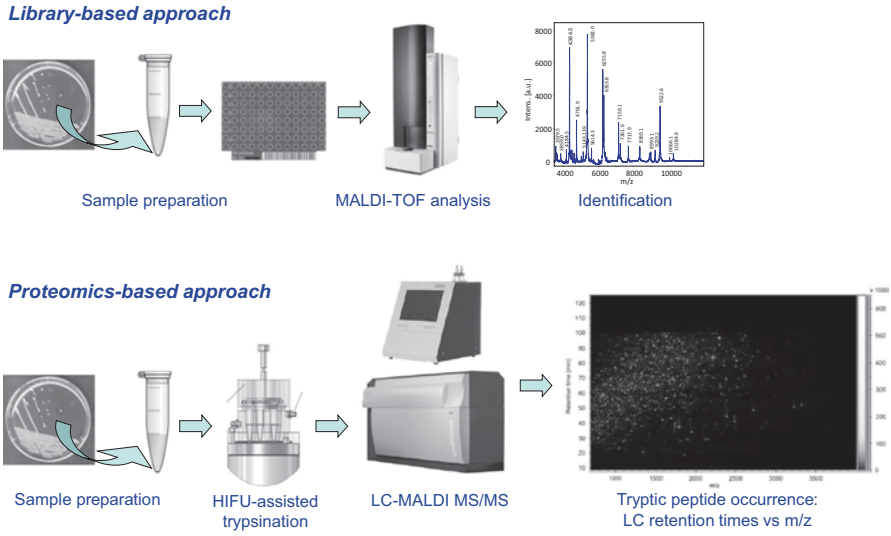

Fig. 11.1 Classical library-based approach for identification of bacteria using MALDI-TOF MS *(top)* and workflow for the novel proteomics-based approach for identification using HIFU-assisted trypsination and LC-MALDI MS/MS *(below)* as described in detail in Sect. 2 of this chapter

subspecies: *arizonae, enterica,* and *houtenae*. It is, to the best of our knowledge, the first study using the classical MALDI biotyping extract directly for proteomic analyses in a bottom-up approach. This rapid procedure allows generation of tryptic peptides within minutes without need for any further processing straight from a simple whole-cell extract. Details of the experimental procedure and data analysis allowing identification of unique biomarker peptides for subspecies discrimination shall be given in the next section.

Experimental Procedure

After selecting a representative strain for each of the subspecies to be discriminated within the genus of interest (e.g., *Salmonella*), a classical MALDI biotyping extraction is performed for each subspecies using a simple acid/organic solvent extraction as previously described (Sauer et al. 2008). We recommend using at least three biological replicates per subspecies. For extraction, an overnight culture of each strain is diluted to $OD_{600\ nm} = 1$. One milliliter of each diluted culture is centrifuged at $5000 \times g$ for 1 min, the medium is discarded, and the resulting pellet is washed with deionized water. The resulting cell suspension is spun again and the pellet is resuspended in 75% ethanol. After centrifuging the sample for 2 min at $16{,}100 \times g$, the supernatant is discarded and the residual ethanol is allowed to evaporate for 5 min at room temperature. In the following steps, the proteins are extracted by consecutive addition and vortexing in 100 µl of 70% formic acid and 100 µl of 100% acetonitrile. Finally, after another spin ($16{,}100 \times g$, 2 min) the supernatant containing the extracted proteins is transferred to a fresh Eppendorf tube where the extract can be stored at −20 °C until further analysis.

In order to generate tryptic digests, each extract should first be dried completely using a SpeedVac concentrator (room temperature, approximately 30–45 min). The remaining pellet which is hardly visible is then resolubilized in 3 µl of 100% acetonitrile, 3 µl of RapiGest, 18 µl of 100 mM Tris-HCl (pH 8.2), and 3 µl of 1 M Tris-HCl (pH 8.2) using 5 min HIFU treatment (UTR200, Hielscher Ultrasonics, Teltow, Germany) (intensity 90%, cycles 0.8). Trypsin (0.1 mg/ml in 10 mM HCl) is then added and the extracted proteins are digested within only 15 min under HIFU treatment, which significantly accelerates the generation of peptides otherwise lasting up to 2–16 h depending on the incubation temperature. Up to six samples can be processed simultaneously. In order to avoid a drastic temperature increase, HIFU treatment should always be performed in an ice water bath. Each digest is subsequently spotted onto a MALDI target (e.g., MTP AnchorChip 1536 TF) using a nano-LC system coupled to a fraction collector. The eluting peptides are directly mixed in the fraction collector with a matrix solution containing α-cyano-4-hydroxycinnamic acid (CHCA). One per eight spots can be manually spotted with a peptide calibration standard diluted in CHCA matrix at a ratio of 1:200. Details of the system, reagents, and operation parameters are given in Table 11.2.

Table 11.2 System requirements, reagents, and operation parameters for nano-LC spotting of tryptic digests onto the MALDI target for MALDI-TOF/TOF MS analysis

nano-LC column	15 cm × 75 µm C18 column; particle size 3 µm, pore size 100 Å
Trap column	2 cm × 100 µm C18 reversed phase column (solid phase extraction); particle size 5 µm, pore size 120 Å
Reagents	
CHCA tryptic digest	748 µl acetonitrile–water–TFA (95:4.9:0.1, vol/vol/vol) 36 µl saturated CHCA (10 mg/ml) in acetonitrile–water–TFA (90:9.9:0.1, vol/vol/vol) 8 µl of 10 % TFA 8 µl of 100 mM $NH_4H_2PO_4$ dissolved in water
CHCA peptide calibration standard	748 µl acetonitrile–water–TFA (85:14.9:0.1, vol/vol/vol), rest as for CHCA tryptic digest
Mobile phase A	0.1 % TFA in water
Mobile phase B	0.1 % TFA in 90 % acetonitrile
Operation parameters	
Spotting interval	Six spots per minute
Elution gradient	Linear, mobile phase B from 2 to 45 %, 64 min, 300 nl/min

After having spotted the tryptic digests, the MALDI-TOF/TOF MS spectra are acquired. The obtained data are then searched on the MASCOT search engine using the National Center for Biotechnology Information (NCBI) or UniProtKB/SwissProt database. The latter should be preferred because of the higher reliability of its reviewed data entries; however, one has to make sure that it contains a satisfactory number of entries for the subspecies under investigation. The NCBI database on the other hand will yield more potential biomarker peptides than the UniProtKB/SwissProt database search. To limit the amount of irrelevant peptide matches, the search can be restricted to the genus under investigation. A peptide decoy database and the MASCOT Percolator algorithm may be used to increase the significance of search results. Further suggested acquisition and search parameters are listed in Table 11.3.

The next step is the search for potential biomarker peptide masses, for example, by applying a Microsoft Excel macro to the data (as described by Gekenidis et al. 2014). The MALDI-TOF/TOF measurement yields compound lists for each digest containing the masses (*m*/*z* values) of all the peptides measured as well as the corresponding signal-to-noise (*S*/*N*) ratios. A biomarker peptide mass is defined as an *m*/*z* value present in all sets of one subspecies but absent from all sets of the others. To identify potential biomarker *m*/*z* values, all lists obtained for the analyzed subspecies and biological replicates are merged in one Excel table and sorted by increasing *m*/*z* values after having added a tag to each entry (see Table 11.4). Then, the *S*/*N* ratio and a ppm value are defined. Note that the *S*/*N* ratio should be equal to or above the value defined for MS/MS precursor selection (see Table 11.3), whereby only masses for which an MS/MS spectrum was acquired will be considered. The macro will select datasets with an *S/N* ratio equal to or greater than the defined value and

Table 11.3 Parameters for MALDI-TOF/TOF MS data acquisition and peptide search

MS/MS precursor selection	Signal-to-noise ratio threshold 10
Compound merging	When separated by less than six fractions, mass tolerance ± 50 ppm
Measurement settings	Laser frequency 1000 Hz Positive reflectron mode Acquisition range 700–4000 Da 3000 shots per spot, 100 shots per raster spot Laser intensity and detector sensitivity: highest peak 10^4–10^5 arbitrary units
Peptide search	
Search restrictions	Tryptic peptides with variable methionine, histidine, tryptophan oxidation One miscleavage Peptide tolerance ± 50 ppm, peptide charge + 1 MS/MS tolerance ± 0.7 Da

Table 11.4 Extract of compiled compound tables from *Salmonella enterica* subsp. *arizonae, S. enterica* subsp. *houtenae,* and *S. enterica* subsp. *enterica* prepared for biomarker peptide mass search (raw data from Gekenidis et al. 2014)

Tag	*m/z*	*S/N*
enterica_a	2703.26023	6.4
enterica_b	2703.27328	4.2
enterica_a	2703.28559	9.0
houtenae_a	2703.29299	10.0
enterica_b	2703.29761	6.8
arizonae_a	2703.30283	5.5
enterica_b	2703.30725	5.6
enterica_b	2703.30853	7.8
houtenae_b	2703.30992	6.8
enterica_a	2703.31167	8.6
arizonae_a	2703.31628	7.4
houtenae_c	2703.32243	8.5
enterica_b	2703.32295	4.4
houtenae_c	2703.33365	106.7
houtenae_a	2703.33666	14.3
arizonae_b	2703.33781	3.9
enterica_b	2703.33957	3.3
enterica_b	2703.34086	4.0
arizonae_a	2703.37732	3.0
houtenae_b	2703.37971	33.7

create a pivot table containing the *m*/*z* values in the row fields and the tag of the different measurements in the column fields. Each *m*/*z* value is then taken as a center, *m*/*z* values within the surrounding ppm window are counted, and the counts of the tag of the different measurements are recorded in the pivot table (see Table 11.5). Such a pivot table will give an overview of the number of *m*/*z* signals within the

Table 11.5 Extract of the pivot table generated from data on *Salmonella enterica* subsp. *arizonae, S. enterica* subsp. *houtenae,* and *S. enterica* subsp. *enterica* (*S/N* = 10, ± 100 ppm). Each *m/z* value is used as center of a ppm window to search for adjacent *m/z* values (see the text). Bold numbers indicate to which biological replicate the respective *m/z* value belongs. Two potential biomarker peptides are highlighted in green and purple (raw data from Gekenidis et al. 2014). In the case of the potential biomarker peptide highlighted in green, four *m/z* values were observed in the three biological replicates of *S. enterica* subsp. *houtenae* (2703.29299 and 2703.33666 in *houtenae_a,* 2703.33365 in *houtenae_b,* and 2703.37971 in *houtenae_c*), but none in the other two subspecies. The average of those four *m/z* values was therefore defined as a potential biomarker peptide mass for *S. enterica* subsp. *houtenae*.

m/z	*arizonae_a*	*arizonae_b*	*arizonae_c*	*houtenae_a*	*houtenae_b*	*houtenae_c*	*enterica_a*	*enterica_b*	*enterica_c*
2701.34674	1	**2**	1	1	2	1	1	2	2
2701.34875	1	2	1	1	2	1	1	**2**	2
2701.39451	1	2	1	1	2	1	**1**	2	2
2702.26381	2			1				**1**	
2702.27090	2			**1**				1	
2702.27861	**2**			1				1	
2702.30037	**2**			1				1	
2703.29299				**2**	1	1			
2703.33365				2	**1**	1			
2703.33666				**2**	1	1			
2703.37971				2	1	**1**			
2704.23789	1	1	**1**						
2704.28700	**1**	1	1						
2704.29833	1	**1**	1						
2705.06338		2	**2**		1	1		1	1
2705.06406		**2**	2		1	1		2	1
2705.13323		2	2		1	**1**	2	3	2

ppm window per set of each subspecies. An extract of a macro used for creation of such a pivot table is given in Fig. 11.2. After having generated the pivot table, the *m/z* values of biomarker peptides are selected and pasted into a new Excel sheet (for examples of such biomarker peptide masses, see marked entries in Table 11.5). Finally, all potential biomarker peptide masses are compared to the results obtained from the MASCOT search in order to find the masses belonging to actual peptides in proteins of the investigated genus. For those actual peptides, a sequence comparison can be made between the biomarker peptide and the corresponding peptides of

```
…
RawSheet = ActiveSheet.Name
NrRows = ActiveSheet.UsedRange.Rows.Count
NrColumns = ActiveSheet.UsedRange.Columns.Count

…
'Determine start row
For Z = 3 To NrRows
    If (Cells(Z, MZColumn) - Cells(2, MZColumn)) > (MZWindow * Cells(Z, MZColumn).Value /
2)
Next Z
StartRow = Z - 1

'Determine end row
For Z = 1 To (NrRows - 1)
    If (Cells(NrRows, MZColumn) - Cells((NrRows - Z), MZColumn)) > (MZWindow *
Cells((NrRows - Z), MZColumn).Value / 2) Then Exit For
Next Z
EndRow = NrRows - Z

'Row-by-row processing of data
PivotRow = 1
For Z = StartRow To EndRow

'Check S/N limit: jump to next row if S/N ratio lies below defined limit
    If Cells(Z, SNColumn).Value < SNLimit Then GoTo NextZ
    PivotRow = PivotRow + 1
    Tag = Sheets(RawSheet).Cells(Z, TagColumn).Value
    For i = 2 To NrColumnsPivot
        Select Case Tag
            Case Pivot(i)
                PivotColumn = i
                Exit For
        End Select
    Next i
    Sheets(PivotSheet).Cells(PivotRow, 1).Value = Sheets(RawSheet).Cells(Z,
MZColumn).Value
    Sheets(PivotSheet).Cells(PivotRow, PivotColumn).Value = 1 +
Sheets(PivotSheet).Cells(PivotRow, PivotColumn).Value
    Sheets(PivotSheet).Cells(PivotRow, PivotColumn).Font.Bold = True

'Analyze ppm window from center to upper m/z limit
    For O = (Z + 1) To NrRows

'Check S/N limit
        If Cells(O, SNColumn).Value < SNLimit Then GoTo NextO

'Check whether m/z value lies within ppm window (upper half)
        If (Cells(O, MZColumn) - Cells(Z, MZColumn)) > (MZWindow * Cells(Z,
MZColumn).Value / 2) Then Exit For
        Tag = Sheets(RawSheet).Cells(O, TagColumn).Value
        For i = 2 To NrColumnsPivot
            Select Case Tag
                Case Pivot(i)
                    PivotColumn = i
                    Exit For
            End Select
        Next i
        Sheets(PivotSheet).Cells(PivotRow, PivotColumn).Value = 1 +
Sheets(PivotSheet).Cells(PivotRow, PivotColumn).Value
NextO:
    Next O

'Analyze ppm window from center to lower m/z limit
    For U = 1 To (Z - StartRow)

'Check S/N limit
        If Cells(Z - U, SNColumn).Value < SNLimit Then GoTo NextU
'Check whether m/z value lies within ppm window (lower half)
        If (Cells(Z, MZColumn) - Cells((Z - U), MZColumn)) > (MZWindow * Cells(Z,
MZColumn).Value / 2) Then Exit For
        Tag = Sheets(RawSheet).Cells((Z - U), TagColumn).Value
        For i = 2 To NrColumnsPivot
            Select Case Tag
                Case Pivot(i)
                    PivotColumn = i
                    Exit For
            End Select
        Next i
        Sheets(PivotSheet).Cells(PivotRow, PivotColumn).Value = 1 +
Sheets(PivotSheet).Cells(PivotRow, PivotColumn).Value
NextU:
    Next U

NextZ:
      Next Z
```

Fig. 11.2 Extract of the Excel macro code used for determination of subspecies-specific peptide masses as applied to process raw data from Gekenidis et al. (2014)

the other subspecies in order to identify the amino acid exchanges responsible for the uniqueness of each biomarker peptide.

Identification of Antibiotic Resistance Mechanisms in Bacteria Using Tryptic Peptides

Since their discovery, antibiotics have been extensively used to fight bacterial infections. This broad use has led to a drastic increase in the occurrence of antibiotic-resistant bacteria, representing one of the major current threats to human health. Consequently, there is a need for discovery of novel antibiotics or drug targets. Identification of the pathways involved in resistance and understanding the underlying molecular mechanisms are important steps toward fulfilling this task.

A recent application of MALDI-TOF MS focuses on the elucidation of resistance mechanisms in bacteria, involving mainly resistance to antibiotics. An extensive recent review on the application of MALDI-TOF MS for detection of antibiotic resistance mechanisms has been published by Hrabak et al. 2013. In the following we shall focus on studies using gel electrophoresis followed by tryptic protein digestion to (a) elucidate changes in expression profiles associated with exposure of microorganisms to antibiotics, (b) identify the proteins being up- or down-regulated as a consequence of antibiotic exposure, and (c) deduce mechanisms involved in bacterial resistance. The vast majority of these studies rely on a principle described as early as 1996 by Shevchenko for in-gel tryptic digestion and mass spectrometric sequencing of proteins (Shevchenko et al. 1996).

Decreased membrane permeability is one major mechanism providing antibiotic resistance to bacteria. In 2001, Dé and coworkers investigated the role of the major porin in *Enterobacter aerogenes* for its resistance to cephalosporins (Dé et al. 2001). They first purified the porin and observed a mass difference in the wild-type and resistant strain porins by MALDI-TOF MS which they hypothesized to rely on a difference in the primary sequence. The SDS-PAGE protein bands of the two proteins were then digested in-gel with trypsin and peptide mapping by MALDI-TOF and nanospray MS/MS identified a G to D mutation in one of the porin's loops, which was suggested to be conferring antibiotic resistance to the clinical *E. aerogenes* strain.

Conejo et al. reported the loss of the outer membrane porin protein D (OprD) in *Pseudomonas aeruginosa* in response to zinc eluting from siliconized latex urinary catheters (SLUC) resulting in carbapenem resistance (Conejo et al. 2003). The outer membrane proteins of *P. aeruginosa* grown in the presence and absence of zinc were prepared, and the expression profiles were compared after separation by SDS-PAGE. Further analysis by MALDI-TOF MS after in-gel tryptic digestion of outer membrane proteins not expressed in the zinc-supplemented extract (and the SLUC eluate) revealed that they matched OprD. The authors concluded that the loss of OprD from *P. aeruginosa* in the presence of zinc is the underlying mechanism for the previously reported increased resistance of this bacterium to imipenem, an antibiotic belonging to the class of carbapenems.

Recently, Khatua et al. elucidated a novel mechanism for how sialic acids on OprD might confer β-lactam antibiotic resistance to *P. aeruginosa* (Khatua et al. 2014). Strains containing α2,3- and α2,6-linked sialic acids have previously been shown to have increased resistance to β-lactam antibiotics. Therefore, after purifying sialoglycoproteins from the membrane fractions of four clinical *P. aeruginosa* isolates and separating them by 2D gel electrophoresis, Khatua et al. digested those proteins in-gel with trypsin and analyzed the resulting peptides by MALDI-TOF/TOF MS. Sialoglycoproteins containing either α2,3-, α2,6-linkages, or both could be identified, among others an OprD precursor. In a subsequent step, sialylated OprD proteins were purified by anion exchange chromatography, and their identity was confirmed by trypsin digestion and MALDI-TOF/TOF MS. Further experiments led the authors to the conclusion that sialic acids on the OprD protein hampered its interaction with β-lactam antibiotics, probably thereby increasing the survival of such strains under antibiotic pressure.

Another study on *P. aeruginosa* (Peng et al. 2005) examined the sarcosine-insoluble outer membrane fraction upon treatment with ampicillin, kanamycin, and tetracycline to identify proteins related to the respective antibiotic resistances. The authors found 11 differential proteins, which were excised from the 2D gel and identified by MALDI-TOF MS after in-gel tryptic digestion of the excised spots. Apart from some known antibiotic resistance proteins, Peng et al. discovered some new proteins and thereby novel potential antibiotic targets.

The same technique of in-gel tryptic digestion and subsequent identification by MALDI-TOF MS was applied by Dupont and coworkers in *Acinetobacter baumannii,* an opportunistic bacillus comprising increasing numbers of resistant strains (Dupont et al. 2005). They compared the outer membrane of different strains and found two differentially expressed proteins, one of which was identified as belonging to the OprD family.

A final example demonstrating the importance of membrane permeability as a mechanism of antibiotic resistance is a study from 2006 which investigated the response of outer membrane proteins in *E. coli* to tetracycline and ampicillin (Xu et al. 2006). Three known and six new outer membrane proteins related to antibiotic resistance in *E. coli* K-12 could be identified.

Another set of studies explored changes occurring in the overall proteome in response to antibiotic treatments (Cordwell et al. 2002). Cordwell et al. used 2D gel electrophoresis to compare the protein profiles of an MSSA and an MRSA strain. A total of 377 proteins were analyzed by MALDI-TOF MS following tryptic digestion of gel-purified proteins. Proteins which could not be identified by MALDI were subjected to tandem electrospray ionization (ESI) MS. In addition, the effect of Triton X-100, a detergent known to reduce methicillin resistance, was investigated. Here, 44 proteins showed altered abundance on the 2D gel with 11 spots found exclusively in the resistant strain. Based on these findings, the authors could conclude that among other factors, products of the σ^B and the SarA regulon (the alternative sigma factor and a regulator of virulence genes) are involved in methicillin resistance of *S. aureus*.

Another study by Cho et al. on MRSA investigated the effect of tea polyphenols (TPP) on the protein expression of a clinical MRSA isolate displaying an excellent synergistic effect of TPP and oxacillin (Cho et al. 2008). Down-regulation of 14 extracellular proteins (chaperone-like and other proteins related to cellular pathogenicity mechanisms as identified by MALDI fingerprinting) and up-regulation of 3 proteins upon TPP exposure were observed. Although the underlying mechanism for this synergy of TPP and oxacillin could not be elucidated, the findings show a clear effect of TPP on the expression of several key MRSA proteins.

Eyraud et al. could show how a small regulatory RNA, SprX, influences antibiotic resistance of *S. aureus* to two glycopeptides, vancomycin and teicoplanin, which are the antibiotics of choice to treat MRSA infections (Eyraud et al. 2014). By constructing a mutant strain lacking expression of SprX (Δ*sprX*) and comparing its expression profile with the wild-type, the authors could identify a SprX target, stage V sporulation protein G, SpoVG, which is significantly down-regulated in the presence of SprX. Of note, SpoVG has been suggested previously to fulfill more general regulatory functions unrelated to sporulation in nonsporulating bacteria such as *S. aureus* (Meier et al. 2007; Schulthess et al. 2011).

Other studies have conducted 2D gel electrophoresis and tryptic peptide-based proteomic surveys on *Mycobacterium tuberculosis*. Sharma et al. analyzed whole-cell extracts of streptomycin-susceptible and streptomycin-resistant clinical isolates of *M. tuberculosis* (Sharma et al. 2010). In 2013, Kumar and coworkers could identify 12 proteins consistently up-regulated in resistant isolates (Kumar et al. 2013). Finally, Truong et al. published results on expression changes related to proteins associated with resistance to rifampicin and isoniazid (RH), the key drugs for tuberculosis treatment (Truong et al. 2014). A comparison of the proteome extracted from RH-resistant and RH-susceptible clinical isolates after 2D gel electrophoresis separation yielded 41 spots with differential expression. After identification of the corresponding proteins by MALDI-TOF/TOF MS analysis of the generated tryptic peptides, 12 proteins involved in virulence, adaptation, and lipid metabolism were identified.

Recent investigations on a cefotaxime-resistant *E. coli* strain WA57 (producing extended-spectrum β-lactamase) revealed 40 differentially expressed proteins from different cell compartments (extracellular, periplasmic, cytoplasmic, membrane, and whole-cell) upon exposure to cefotaxime (Gonçalves et al. 2014). These 40 and additional 275 proteins were all identified by analyzing tryptic protein digests with MALDI-TOF/TOF MS. This study gives a comprehensive overview of the changes occurring in the *E. coli* strain WA57 when stressed with cefotaxime. Chaperone, porin, and export proteins were particularly affected, suggesting an important role of stress response and transport functions in antibiotic resistance of this strain.

Another important principle underlying antibiotic resistance is the inactivation of the agent by either chemically modifying it (e.g., hydrolysis) or directly binding to it (antibiotic trapping) (Goessens et al. 2013). Goessens et al. hypothesized a covalent binding of meropenem to an enzyme in *E. coli* as an underlying resistance mechanism toward carbapenems. A comparison of a carbapenem-susceptible *E. coli* to its carbapenem-resistant successor strain isolated from the same patient

after carbapenem treatment showed that the resistant strain additionally possessed the plasmid-encoded β-lactamase CMY-2. In order to confirm their hypothesis of an acyl–enzyme complex formation, they incubated periplasmic extracts with meropenem, separated those as well as untreated extracts on SDS–PAGE, and analyzed the excised CMY-2 band on a MALDI-TOF/TOF MS after in-gel digestion with trypsin. By comparing the tryptic peptides in which the active site of the enzyme is located from treated and untreated samples, they found the peptide mass corresponding to the peptide containing the active site after modification with meropenem and removal of an acetaldehyde group in the samples treated with meropenem. This finding strongly supports the hypothesis of meropenem being covalently bound to CMY-2 as a possible antibiotic resistance mechanism.

A recent study from 2014 exploited the analysis of in-gel trypsin-digested proteins with MALDI-TOF/TOF MS to identify CMY-2-type cephalosporinases in Enterobacteriaceae (Papagiannitsis et al. 2014). A peak uniquely observed in CMY producing isolates was thereby confirmed to represent a *C. freundii*-like β-lactamase.

In a comparative proteome study with a multi-resistant *E. coli,* 21 differentially expressed proteins under treatment with multiple drugs were identified (Piras et al. 2012). From the identified proteins, the authors concluded that quorum sensing might be involved in the multiple antibiotic resistance observed in this strain.

Hemmerlin et al. investigated by applying 2D gel electrophoresis, tryptic digestion, and MALDI-TOF MS the effect of fosmidomycin on *E. coli* being only shortly exposed to the antibiotic (Hemmerlin et al. 2014). Within the first 3 h after exposure, combined strategies are triggered mainly consisting of adapting metabolism to increase tolerance to oxidative stress and rapidly exporting the antibiotic from the cell. Such insights can aid the development of new efficient drugs by improving the understanding of the underlying defense mechanisms.

Similar studies can be conducted using mass spectrometric methods other than MALDI-TOF. However, being beyond the scope of this brief overview, we shall mention only one study from 2007 by Camara and Hays (2007). A protein with an approximate mass of 29,000 Da found only in ampicillin-resistant *E. coli* was confirmed to be a β-lactamase by in-gel digestion followed by liquid chromatography-mass spectrometry (LC-MS).

Another approach, circumventing the time-consuming preparation of 2D gels, was shown by Wilcox et al. (2001). Instead of digesting protein bands separated on a gel, they analyzed tryptic digests of fractions collected from an HPLC. In particular, three ribosomal proteins responsible for streptomycin, erythromycin, and spectinomycin resistance in three *E. coli* strains were investigated. The mutations responsible for the observed resistance were located by analyzing tryptic peptides on a MALDI-TOF/TOF and a nano-electrospray tandem mass spectrometer.

In conclusion, tryptic digestion of proteins and analysis of the resulting peptides by MALDI-TOF MS or LC-MS have proven to be a potent tool to elucidate mechanisms underlying bacterial resistance to antibiotics. Either proteins of interest or protein fractions such as outer membrane proteins can be analyzed, or the whole proteome of antibiotic-susceptible and -resistant strains can be compared. The proteins of interest or the differentially expressed proteins can be digested to

tryptic peptides and then further analyzed. New proteins related to resistance are thereby identified, and mutations responsible for resistance can be located. Resistance mechanisms are usually deduced from the function of the identified proteins. In the case of antibiotic trapping as the underlying mechanism, that is, the covalent binding of the antibiotic to a target, the actual antibiotic–target complex can be detected. The number of analyzed proteins going up to several hundreds and the broad spectrum of antibiotics and bacteria tested in the aforementioned studies show the global applicability of these approaches.

Limitations and Future Perspectives

Intact protein expression typing of pathogenic bacteria has been continuously improved since in the mid-eighties MALDI-TOF MS and ESI MS have been established as efficient soft-ionization techniques of biomolecules. For rapid identification and classification, the intact protein MALDI-TOF MS approach has to be favored as compared to the ESI MS technique with respect to reproducibility, speed, and robustness of data acquisition, and cost-effectiveness. However, in cases where time to result and increased complexity of analytical workflows and infrastructures are not of primary concern, state-of-the-art technologies aiming at profiling bacteria according to their peptide profiles (proteomics-based approach) give preference clearly to the ESI MS method. Even in the light of the many achievements and advantages of whole-cell mass spectrometry (biotyping), there exist currently still limitations and areas that need to be improved in the future, such as the following:

- Accurate identification of strains (below-species level) for the precise definition of organisms and communities under investigation
- Identification of single or multiple bacteria in bacterial mixtures, opening up a wide range of possibilities to investigate diversity at the biologically relevant level
- Identification without *in vitro* culturing, directly from environmental samples contaminated by pathogenic bacteria or identification of non-culturable bacteria (non-culture-based identification)
- Set-up and publish lists of organism-specific biomarkers (proteins or peptides) as a community resource for quick and reliable identification
- Targeted proteomics approaches using selected reaction monitoring (SRM) methods for increased accuracy and sensitivity in quantification
- Merging proteomic and genomic databases, using so-called proteogenomics approaches allowing to identify and characterize previously undescribed species and proteins
- Enrichment of bacteria applying bead-based technology prior to identification to increase analytical sensitivity, particularly from low abundant specimens

Direct and rapid identification of bacteria from environmental samples and mixtures by MALDI-TOF MS remains challenging. Target bacteria may exist in low

concentrations (below the analytical detection limit) and background from the sample matrix may influence subsequent analyses. As an approach to separate and enrich target bacteria without culturing using standard microbiological enrichment procedures, functionalized magnetic nano-beads, for example, coated with antibodies, are promising for preparation of such samples prior to MALDI-TOF MS analysis (Ho et al. 2004; Schlosser et al. 2007; Madonna et al. 2001). Aiming to implement such approaches in routine diagnostics, future work needs to emphasize increasing cell recovery rates as well as overcoming cross-reaction with non-target bacteria and agglutination of beads. In particular, success in these fields will strongly depend on the development and application of new specific affinity probes.

In order to increase the discriminatory power of MS-based methods of bacterial characterization, we have been investigating into a discovery-based proteomics approach making use of the traditional organic acid/organic solvent extracted biotyping sample (Gekenidis et al. 2014; Drissner et al. 2014). As commented in the sections above, we could clearly demonstrate that this ready-to-use, straightforward preparation—free of any contaminating ingredients, such as detergents—is proving to be a very valuable starting material to perform proteomics experiments using LC-MALDI MS. We have now compared the equivalent tryptic digests with LC-ESI MS and found that, by combining both ionization techniques, the information content regarding the produced peptides significantly increased (unpublished results). The ultimate aim of generating such biomarker peptide lists is to switch from discovery-based proteomics into target-based proteomics allowing in the future to monitor pathogenic bacteria with increased discriminatory power using SRM technology. In brief, the mass spectrometer (triple quadrupole) would be set to monitor only selected, microorganism-typic tryptic peptides, and their absolute abundance could be determined by spiking respective isotopically labeled peptides. Until now, there are only a few reports regarding SRM applications in the field of SRM technology of pathogenic bacteria. Aebersold and Picotti have recently paved the way to accurately employ SRM technology within proteomics approaches (Karlsson et al. 2012; Picotti and Aebersold 2012).

In the context of truly real-time detection, it is essential that, as pre-analytical steps, bacterial sample concentration and elimination from the matrix need to be improved and simplified. As recently shown by Barreiro et al. (2012), non-culture-based identification of bacteria in milk by protein fingerprinting is easily performed by using the recently introduced Sepsityper™ by Bruker.

The problem of identifying non-culturable bacteria and the possibility of accurately identifying individual microorganisms out of a mixture have been addressed so far mainly through genomics-oriented projects, for example, by next-generation sequencing applications and with proteogenomics approaches (Pierce et al. 2012; Woo et al. 2014; Lasken and McLean 2014; Sheynkman et al. 2014). The latter one is an area of research interfacing proteomics and genomics and as such helping to identify novel peptides (not present in reference protein sequence databases) from MS-based proteomics data (Nesvizhskii 2014).

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