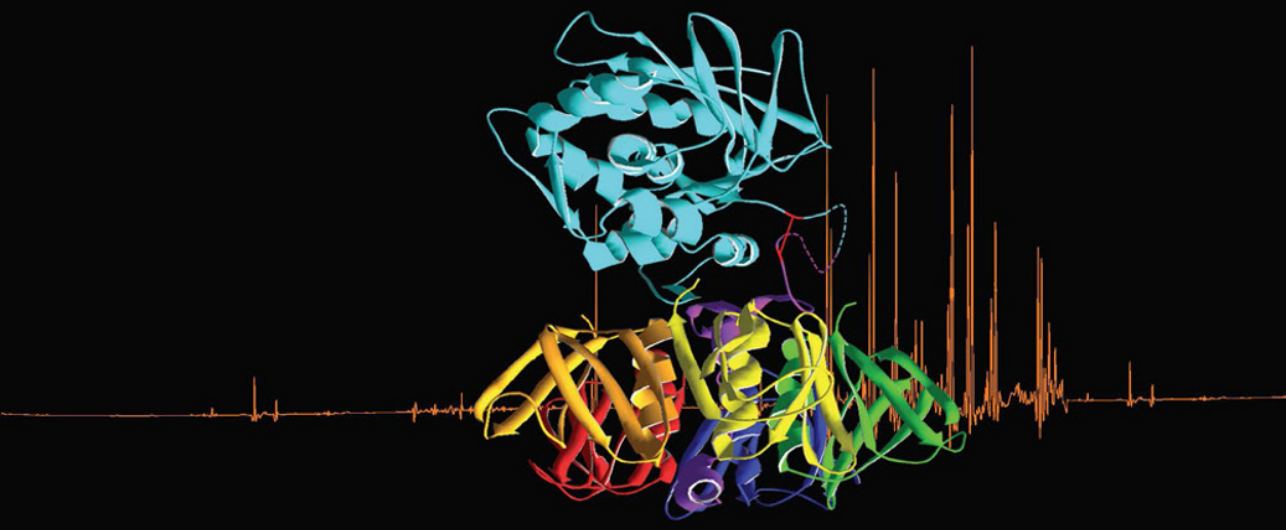
Microbiological Identification using MALDI-TOF and Tandem Mass Spectrometry

Industrial and Environmental Applications



Edited by
Haroun N. Shah • Saheer E. Gharbia • Ajit J. Shah
Erika Y. Tranfield • K. Clive Thompson

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This book is dedicated to the late Professor Franz Hillenkamp who visited and communicated with MISU in the midst of this work and acted as our mentor during the development of MALDI-TOF and tandem mass spectrometry for clinical microbiology

and

Iona, Amaya and Calum.

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Preface

Clinical symptoms provide clues to the aetiology of infections. However, there is no substitute for accurate identification to confirm disease and enable appropriate treatment to be confidently applied. Consequently, microbiological identification has been the cornerstone of clinical microbiology since Gram introduced his differential staining technique in 1884. Along with morphological characters, phenotypic and metabolic fermentation profiles grew in significance and appeared in the first edition of *Bergey's Manual of Determinative Bacteriology* in 1923. This remained the foundation of diagnostic microbiology and the basis for classification and identification of bacteria up until eighth edition of *Bergey's Manual* in 1974. This framework was incorporated into automated and miniaturized commercial kits (e.g. API® ID strip range by bioMérieux) for clinical and subsequently industrial and environmental laboratories to identify microbial species.

In the late 1970s, a new era of mass spectrometry enhanced microbial identification through higher-resolution analysis of microbial cellular components. Extensive analyses of polar and non-polar lipids revealed immense diversity in the microbial kingdom and specific molecular compositions were found to be unique to taxa. A new phase of chemical structure analysis dominated microbial taxonomy and classification and *Bergey's Manual* transitioned to a systematic approach for classification of the microbial kingdom, reflected in a change of the title of the new first edition to *Bergey's Manual of Systematic Bacteriology* (1984). However, implementation of chemical/bioanalytical methods and, in particular, electrophoresis, chromatography and mass spectrometry into diagnostic laboratories remained limited to specialized research laboratories with access to high-resolution analytical resources. Co-editor HNS's laboratory at the Royal London Hospital Medical College, University of London, was one of the pioneering teams to implement chemotaxonomy for the analysis of atypical and metabolically inert bacteria, including anaerobic pathogens. Wider adoption was found too cumbersome and technically demanding and limited their broader endorsement. However, a breakthrough was achieved by gas chromatography of long-chained fatty acid (LCFA) where analysis was partially automated by MIDI Inc., who developed the first dedicated database to drive analysis of lipid profiles. On the other hand, pyrolysis mass spectrometry failed to establish a wider base and was explored mainly by research laboratories. But lack of interlaboratory reproducibility, high cost and their cumbersome nature curtailed its development.

In 1973, Franz Hillenkamp developed a high-performance laser microprobe mass spectrometer with a spatial resolution of 0.5 μm and sub-attogram limit of detection for lithium

atoms. This instrument was commercialized as the LAMMA 500. The more advanced 1000 was one of the first laser desorption mass spectrometers to be used for mass spectrometry imaging of tissues. In 1985, Hillenkamp and Michael Karas used a LAMMA 1000 mass spectrometer to demonstrate for the first time the technique of matrix-assisted laser desorption/ionization (MALDI), which allowed the analysis of large biopolymers. By the mid-1990s several researchers analysed bacterial cells and demonstrated that unique mass spectral profiles could be obtained from different species with MALDI. The concept of exploring the technology for a clinical diagnostic laboratory was not pursued, partly due to a history of failure of mass spectrometry (MS)-based techniques in clinical microbiology.

The UK's Public Health Laboratory Service (PHLS, later PHE and now UK Health Security Agency), a 100-year-old institute that focused on the analysis of pathogens, was structured along the lines of human pathogens/infections; thus a *Staphylococcus* laboratory, enteric or respiratory infections units etc. were led by specialist scientists for principal clinical pathogens. This permitted a high degree of expertise of various pathogens, yet each laboratory still retained a significant level of 'unknown' species in storage that were designated *incertae sedis*. Therefore, in 1997, PHLS established a new laboratory, designated the 'Molecular Identification Services Unit' (MISU), under the directorship of co-editor HNS. The function of MISU was to improve the level of species identification of atypical, rarely isolated and emerging human pathogens through research programmes while providing a more comprehensive diagnostic service function for the organization. Coming from a research background in which 16S rRNA sequence analysis was being developed as a tool for studying microbial phylogenetics, the technique was adopted for bacterial identification and MISU became the first accredited laboratory to implement this approach for human clinical samples. The laboratory also incorporated LCFA profiles as an adjunct to its newly employed 16S rRNA and was therefore well positioned to assess the potential of emerging technologies.

MISU was fortuitously given the opportunity to field-test the first benchtop MALDI-time-of-flight MS (MALDI TOF MS) (Kratos Analytical Inc.) and organized a conference on 27 October 1998 jointly with Kratos Analytical and Manchester Metropolitan University to explore and demonstrate potential applications of MALDI-TOF MS for clinical laboratories. An instrument was placed at the conference lecture theatre and the technique was demonstrated live during the meeting. Its speed of analysis and simplicity had a huge impact on the audience, but the general comment was that it was a new platform for research applications. Undeterred by the negative views, MISU would go on to relentlessly pursue the technology for the next decade for microbial identification of clinical samples and eventually implement it as its frontline approach.

These were interesting times, as concurrently there were two major developments of the technology, one designated surface-enhanced laser desorption/ionization (SELDI)-TOF MS that selectively captured proteins on ProteinChip Arrays prior to MALDI-TOF MS analysis and SEQUENOM's MassArray for genotyping that used reverse transcriptase to produce the more stable RNA for analysis. To our knowledge, MISU was the only laboratory to have implemented the three approaches at the time. The strategy envisioned was that MALDI-TOF MS would be explored for general microbial identification, SELDI-TOF MS for proteotyping of strains, while the MassArray was employed for genotyping of strains by the Genomics, Proteomics and Bioanalytical Laboratory led by co-editor SEG.

Both laboratories jointly organized annual conferences from 1998 to the present to promote and develop new applications of these technologies. These were later supported by co-authors AJS, EYT and KCT until the hiatus caused by the COVID-19 pandemic.

Poor uptake of the ProteinChip and MassArray technologies by microbiologists led to their eventual cessation, while MALDI-TOF MS grappled to gain acceptance. The MS company Micromass (later Waters Inc.) designed the first upright, more compact MS benchtop instrument and was placed at MISU in 2000 to develop the method for clinical microbiology. Because microbial identification was historically based upon patterns of carbohydrate fermentation, the major problem encountered by diagnostic laboratories was the differentiation of non-fermentative species that produced uniformly negative results. However, the introduction of comparative 16S rRNA sequencing permitted insight into the immense diversity of these highly complex microorganisms. Species of the non-fermentative genus *Porphyromonas* that were difficult to distinguish by diagnostic laboratories were used by MISU to establish proof of concept of MALDI-TOF MS for microbial identification. With all 18 species of the genus being unambiguously delineated, work began to standardize protocols and assemble a database using strains from an accredited source, the National Collection of Type Cultures. To assess the potential of MALDI-TOF MS, 16S rRNA and LCFA profiles were included as complementary methods and, by 2004, the first microbial database of 3500 mass spectral profiles were reported and MISU later field-tested the method at the Royal London Hospital. This demonstrated not only the pragmatism of the method but also its value for a clinical laboratory as it was found to be accurate and rapid and offered significantly lower cost.

The annual European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) conferences became the major forum for communications on the development on MALDI-TOF MS. Progress at first was slow and circumspect, with very poor attendance in 2003 in Glasgow to a packed main auditorium capacity in Vienna in 2010 in which data on the success of MALDI-TOF MS to rapidly identify *Clostridium difficile* during an epidemic in UK hospitals were reported by PHE. This congress was a watershed moment for MALDI-TOF MS for several reasons. For example, in the midst of the meeting, bioMérieux entered the field by acquiring the successful diagnostic company AnagnosTec GmbH (Potsdam Golm, Germany), while ThermoFisher announced its engagement with MISU to explore the wider potential of MS for microbial diagnostics. It was also at this meeting that Wiley launched our first book of this series entitled *Mass Spectrometry for Microbial Proteomics* (eds H.N. Shah & S.E. Gharbia), published in 2010. Bruker Microbiology & Diagnostics recognized the impact of dedicated mass spectral profile databases and established a database linked to their specific MALDI-TOF MS for clinical microbiology. They, too, had a significant presence at the Vienna meeting in which there was a dedicated session in MALDI-TOF MS for the first time, entitled 'MALDI-TOF MS in Clinical Microbiology'. PHE's presentation at this meeting, entitled 'MALDI-TOF MS of surface-associated and stable intracellular proteins for identification and resistance profiling of human pathogens' (Shah, H.N., 10–13 April 2010), led to extensive support to expand clinical applications of the technology.

With the London 2012 Olympics approaching and the UK's government keen to establish an economic, accurate and rapid diagnostic method in the event of major outbreaks of infection at the games, six Bruker instruments were placed across PHS's national network

of laboratories in October to deliver rapid local identification of potential biological threats and transmission of infections in mass events. Extended applications from clinical to non-clinical samples were pioneered by ASTA, based in Seoul, Korea, developing specialist databases such as Food, Agriculture and Environmental for specific applications using their own ASTA Tinkerbell LT MALDI-TOF MS instrument. Soon, industrial and environmental applications of MALDI-TOF MS were reported using other MS platforms. A common strategy was to utilize 16S rRNA to delineate new diversity, followed by deposition of the mass spectral data of an unknown isolate into existing databases to expand its capability. Our last conference to promote these technologies prior to the COVID-19 pandemic restrictions was held on 21 and 22 June 2018, entitled 'The Impact of Advances in Mass Spectrometry and Analytical Technologies on Detection and Revealing Microbial Behaviour and Interaction with their Environment', and was co-sponsored by a large number of biotechnology companies such as Ascend Diagnostics, bioMérieux, Inc., Bruker Microbiology & Diagnostics, Shimadzu Corp. and ThermoFisher Scientific.

As microbes continue to be exploited for their industrial and environmental properties, profiling the expressed proteome is now essential for developing commercial applications. For example, an extensive collaborative study, entitled 'Feasibility study to assess the potential of electrospray mass spectrometry to provide mass spectral-based identification to the now established MALDI-TOF MS', which utilized a Q-Exactive, was undertaken between 2012 and 2015. This was reported at ECCMID, Denmark, in 2015, under the title 'A global diagnostic approach for microbial identification: accurate characterisation of difficult to differentiate pathogens', by co-author HNS et al. and demonstrated unambiguous delineation of closely aligned pathogens such as *E. coli* and *Shigella sonnei*. We also reported extensive coverage of the proteome of several human pathogens along with unique strain biomarkers using both bottom-up and top-down methods, some of which were reported in the second book *MALDI-TOF and Tandem MS for Clinical Microbiology* (eds H.N. Shah & S.E. Gharbia), published by Wiley in 2017.

The present book focuses on applications of mass spectrometry in industry and the environment and is divided into four overlapping sections. The first (Chapters 1–5) commences with an historical background leading up to MALDI-TOF MS in the clinical laboratory, including recent viral applications and data analysis such as machine learning algorithms that are being championed for strain typing and tandem MS, involving bottom-up and top-down proteomics. New approaches such as liquid atmospheric pressure and advanced applications in imaging and HDX are proposed for future development. Special attention is given to a new instrument, the Omnitrap, which combines several applications of MS. Chapters 6–10 describe environmental applications as MALDI-TOF MS transitions to non-clinical laboratories. Environmental, agricultural, soil and bioremediation research for typing and biopolymer degradation are considered. Veterinary applications of mass spectrometry for diagnostics are at an early stage and have focused almost entirely on MALDI-TOF MS. Its impact has already been substantial, and Chapters 11 and 12 cover both domestic animals and livestock. Industrial use of MALDI-TOF MS is now very advanced and encompasses a diverse range of applications. Chapters 13–19 report applications in the food, water, marine and pharmaceutical industries, including drug discovery, as well as its potential as part of a contamination control strategy for regulated industries.

1

Progress in the Microbiological Applications of Mass Spectrometry: from Electron Impact to Soft Ionization Techniques, MALDI-TOF MS and Beyond

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1.1 Introduction

Over the past two decades, advances in genomics, proteomics and metabolomics and their key technologies, such as mass spectrometry (MS) and particularly matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS, have propelled microbiology to the forefront of life sciences, radically altering the workflow of diagnostic laboratories and subsequently expanding into environmental and industrial applications. The road map of microbial classification has been profoundly altered and has progressed from a phenotypic, determinative system to one based on phylogeny as new technologies have been incorporated, modified and applied. This transition has been complex, and hence to illustrate its impact, it is discussed here in the first instance in the context of a single genus, *Bacteroides*, first described in 1898 [1, 2]. This is the dominant taxon of the intestinal tract of humans and animals and therefore plays a pivotal role in health and disease. The nature of this multifaceted ecosystem is central to an understanding of the biology of humans and requires in-depth analysis of the physiology and diversity of its microbiome. MS has been the underlying technology used to probe this ecosystem since studies first commenced over six decades ago (see Drasar and Hill [3]).

1.1.1 Algorithms Based upon Traditional Carbohydrate Fermentation Tests

The Gram stain, together with advances in microscopy, built the foundation for the characterization of microbes, dividing the kingdom into four main domains as Gram-negative

Microbiological Identification using MALDI-TOF and Tandem Mass Spectrometry: Industrial and Environmental Applications, First edition. Edited by Haroun N. Shah, Saheer E. Gharbia, Ajit J. Shah, Erika Y. Tranfield, and K. Clive Thompson.

and Gram-positive rods and cocci [4]. Together with morphological criteria, the capacity to ferment/metabolize individual carbohydrates to produce acid was widely adopted for algorithms to identify species and describe new centres of variation and has been reported in various editions of *Bergey's Manual of Determinative Bacteriology* from its first edition [5]. However, the emphasis from its infancy was strongly biased towards clinical applications. Thus, the first diagnostic compendium, titled *Manual for the Identification of Medical Bacteria* [6], utilized these algorithms based upon carbohydrate fermentation tests to delineate clinical isolates to species level. Although this approach expanded and helped to describe new taxa that were saccharolytic or moderately fermentative, non-fermentative species, which represent a significant component of the microbiome of any habitat, remained poorly circumscribed and overlooked through the years. It is among this latter cluster that MALDI-TOF MS would bring about a paradigm shift in clinical microbiology and its eventual transition to non-clinical sites.

1.1.2 Dynamic Changes in the Chemotaxonomic Era (c. 1970–1985) through the Lens of the Genus *Bacteroides*

Genera such as *Bacteroides* that were described decades earlier using the above algorithms accumulated large numbers of species that only loosely fitted their broad definition. With the introduction of DNA analysis in the 1960s (initially as mol% G+C content), this heterogeneity was reflected in their wide range in DNA base compositions (e.g. Owen et al. [7]). The limit of a genus was then fixed at c. 10–12 mol% G+C and was applied primarily as an exclusionary criterion in systematics. *Bacteroides,* with a 28–61 mol% G+C span, was therefore redefined around the type species *B. fragilis* and related taxa with a reduced base composition of c. 40–50% mol% G+C [8] [9], [10]. Consequently, many taxa were left as *incertae sedis* and were subjected to a range of biochemical and chemical analyses that included protein electrophoretic and lipid analyses [11–14]. Within this more restricted definition of the genus *Bacteroides*, three broad groups of species were clearly discernible based on carbohydrate fermentation tests: (i) saccharolytic species, *B. fragilis* group; (ii) moderately/weakly saccharolytic, *Bacteroides melaninogenicus* cluster; and (iii) non-fermentative species, *Bacteroides asaccharolyticus* group (see Figure 1.1).

This heterogeneity presented enormous difficulties, but it was the third group where the largest clinical and taxonomic problems were encountered because of the paucity of reliable characters to define taxa. Members of this group were uniformly non-fermentative but there were indications of heterogeneity using new techniques (Figure 1.1). Thus, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of polypeptides and isoelectric focusing (IEF) of cellular proteins revealed profiles that were concordant with the three groups described earlier (see, e.g., [15, 16]). Multilocus enzyme electrophoresis (MLEE) further corroborated these findings, with the *B. fragilis* group being defined by enzymes of the hexose monophosphate shunt/pentose phosphate pathway in addition to malate and glutamate dehydrogenases, whereas the moderately/nonfermentive groups contained only the last two oxidoreductases [17]. These species, in common with other microorganisms were subjected to extensive lipid analyses using hard ionization techniques in MS, including gas chromatography-MS (GC-MS). Subsequently, the arrival of soft ionization methods such as MALDI-TOF MS in the late 1990s were explored for microbial