

MALDI-TOF MASS SPECTROMETRY OF BACTERIA

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The development of MALDI-TOF mass spectrometry methods for the characterization of bacteria is reviewed and discussed. The general use of MALDI for the characterization of large biomolecules led directly to obvious applications involving the analysis of isolated bacterial proteins. More surprising was the observation that MALDI-TOF mass spectrometry could be applied directly to crude cellular fractions or cellular suspensions and that the resulting data from such complex mixtures could provide evidence for chemotaxonomic classification. Versatility and the rapidity of analysis led to the rapid development of a number of MALDI-TOF methods involving bacteria. Examples of some of the applications covered in this review are the analysis of bacterial RNA and DNA, the detection of recombinant proteins, the characterization of targeted or unknown proteins, bacterial proteomics, the detection of virulence markers, and the very rapid characterization of bacteria at the genus, species, and strain level. The demonstrated capability of taxonomic classification at the strain level, using unprocessed cells, opens the possibility that MALDI-TOF and similar mass spectrometry approaches may contribute significantly to fulfilling emerging needs for the development of near real-time methods for the characterization

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I. INTRODUCTION

This review is intended to cover the characterization of bacteria by matrix-assisted laser desorption-ionization (MALDI) time-of-flight mass spectrometry (TOF/MS), with an emphasis on rapid methods; generally applications that involve whole cells, simple cell lysates, or crude bacterial extracts. A significant coverage of studies that involve a more extensive isolation procedures, or of approaches that rely heavily on other instrumental methods, is not intended. As a consequence, a major theme in this review is the use of MALDI-TOF/MS for bacterial taxonomy. This approach involves the identification (or differentiation) of bacteria based on MALDI-TOF mass spectra for cells or cellular components. Although other well-known methods can also be used to differentiate bacteria, the MALDI-TOF/MS approach has one clear advantage, speed of analysis. Chemotaxonomy of bacteria by MALDI-TOF/MS can be accomplished rapidly, and many of the methods described below can

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be completed within a few minutes, provided that sufficient bacteria have been collected prior to mass spectral analysis. Usually, but not always, bacteria are grown via culture methods prior to analysis. Although this factor might be considered a disadvantage, it should be noted that competing molecular biology methods also require preliminary culture steps prior to their use. With respect to information content, the MALDI spectra of bacteria can be considered to be at least complementary to data obtained from other methods for bacterial identification. Moreover, MALDI provides unique insights into bacterial biology and chemistry based on the detection of specific chemicals or in the measurement of chemical changes in response to environmental, temporal, or other external influences. This review includes a sampling of studies that involve the detection of isolated bacterial proteins (e.g., bacterial toxins), bacterial DNA or RNA, and bacterial metabolites. Generally, studies are cited from those categories that involved the analysis of the bacterial components obtained by using only simple and rapid methods for isolation or cleanup in the steps prior to MALDI-TOF/MS analysis. Although the analogous characterization of other microorganisms is beyond the scope of this review, similar studies of fungi (Welham et al., 2000) and viruses (Tan, Wong, & Kini, 2000) by MALDI are also beginning to appear in the literature.

Shortly after the introduction of MALDI as a mass spectrometry ionization technique, it began to be used for the study of bacterial biopolymers. These early studies involved the analysis of marker analytes (biomarkers) after isolation from bacterial cells. In more recent studies, biomarkers have been detected directly from the organism itself. A few studies have been undertaken to explain the reactivity and toxicity of specific chemicals. Such studies can address questions that involve either bacterial or other cellular-toxicity endpoints because bacteria can be used to model toxicity and metabolism in other organisms. For example, in the alkylation of a model *Escherichia coli* protein, thioredoxin was used to explain the increased toxicity of a glutathione conjugate of a dihaloethane (Meyer et al., 1994). The unusual toxicity of the glutathione conjugate (glutathione conjugation usually detoxifies compounds) is attributed to the fact that the dihaloethane-glutathione molecule is able to alkylate the protein thioredoxin, and it presumably would also alkylate other proteins as well.

Typically, most early MALDI-TOF/MS studies of bacteria involved *E. coli*, a common bacterium that continues to be one of the most frequently studied of microorganisms today. Although the most frequently measured biomarkers in MALDI studies of bacteria have been proteins, there have been a few reports on the analysis of bacterial RNA or DNA. Indeed, one of the first applications of MALDI to bacteria involved the analysis

of bacterial RNA. A study by Nordhoff et al. (1992) reported the analysis of RNA isolated from *E. coli*. Other studies of bacterial RNA or DNA are detailed in Section II. As noted above, more commonly proteins were the bacterial biopolymers studied with MALDI. In the earliest experiments, they were isolated from bacteria and analyzed in concentrated/purified form, using MALDI methods already being developed for the general analysis of proteins after isolation and purification. There are now a large number of studies that report on the characterization of specific proteins from *E. coli* and other organisms. A significant number of these studies involved the detection of recombinant proteins expressed in bacteria (Section III).

A description of the more general applications of MALDI-TOF/MS to the identification of unknown bacterial proteins is described in Section IV. That section includes brief accounts of the discovery of a few previously uncharacterized biomarker proteins, including toxins, virulence factors, and strain-specific markers. As larger numbers of proteins have been identified in studies, a proteomics approach has developed as bacterial proteins have been examined in groups, and as their relationships one to another have been monitored (Section V). Other applications that involve the analysis of proteins isolated from bacteria are included in Section VI. That section includes two unusual applications that do not easily fit into any of the other sections. One is the reported detection of non-covalent association between bacterial proteins by MALDI-TOF/MS, whereas the other is a study of carbohydrate metabolism in bacteria. The development of methods for the characterization of whole cells, with little or no sample pretreatment, is covered in Sections VII–IX. The capability to rapidly analyze whole cells has led to a number of studies that demonstrate the possibilities for the rapid identification of specific organisms or stains. Such studies are possible because the biological variation that occurs at the strain level can be significant enough to produce unique biomarker (protein) profiles that can be readily detected by MALDI-TOF/MS, even when no specific steps are taken to enrich samples in the strain-specific proteins. The rapidity of the MALDI method, the sometimes single-strain specificity of organisms that have human health effects, and the absence of methods for rapidly differentiating among similar strains, together provide a compelling basis for the development of rapid methods for strain-specific taxonomic identification of bacteria. Thus, studies that involve rapid bacterial identification, typically using whole cells, are covered to the greatest extent in this review. MALDI seems uniquely suited to the task of rapidly characterizing intact organisms, based on the rapid detection of a unique bacterial fingerprint at the genus, species, strain, and even the sub-strain level.

II. BACTERIAL RNA AND DNA

The amplification of DNA, using polymerase chain reaction (PCR) methods, provides a means of producing large quantities of DNA from a relatively small number of organisms. Traditional culture methods often lack the sensitivity and specificity that can be attained with the PCR method to amplify targeted regions of the bacterial genome. It is not unexpected then, that MALDI-TOF mass spectrometry has been used for the analysis of PCR-amplified DNA in studies that involve bacteria and bacterial genes. In one study, MALDI-TOF mass spectrometry was used for the detection of 108- and 168-base PCR products from *Legionella* after a rapid purification to remove salts and unreacted primers (Hurst et al., 1996). To facilitate the finding of an optimum spot on the sample probe from which the PCR products could be desorbed, a synthetic DNA 20-mer was also added to the sample prior to analysis. Otherwise, the analysis was straightforward. Similarly, products from PCR assays designed to amplify 56- and 99-base regions of the *pmoA* gene from *Methylosinus trichosporium OB3b* and *Methylomicrobium albus BG8*, two species of methanotrophic bacteria relevant to bioremediation, have been reported (Hurst et al., 1998). In these studies, the authors took special note of two obvious factors relevant to the quality of the MALDI spectra from bacterial oligonucleotides; namely, sample cleanup, and the number of bases present in the oligonucleotide. The importance of desalting in the cleanup step was demonstrated with a model oligonucleotide, pdA₄₀-pdA₆₀. The same mixture was analyzed with and without a simple sample cleanup step, using a commercial desalting cartridge. The desalted samples showed ions for the 40-mer through the 50-mer, whereas no analyte ions were detected from unpurified samples. This result explained similar results observed from PCR-amplified bacterial RNA. Regarding the maximum size of the oligonucleotide, the authors reported that it was at or near a 100-mer for the conditions they used. It is also important to note that, although PCR generally produces double-stranded DNA, the MALDI process can dissociate a single product into two single strands, each of which would have its own molecular mass. In the results reported in Hurst et al. (1996), only single-stranded DNA was detected. The detection of double-stranded DNA is given in another example below. In the 2 years between these studies (Hurst et al., 1996, 1998), the sample size necessary for analysis was reduced considerably. In the second study, spectra were obtained from small aliquots (1.5 μ l) from a single 100 μ l PCR mixture, using a typical protocol.

MALDI-TOF/MS has been used to investigate DNA repair mechanisms in bacteria. For example, the mechanism of action of endonuclease III in *E. coli* was studied

recently. The molecular masses of the repaired fragments of 5-OHC- and DHT-containing oligonucleotides were consistent with the theory that endonuclease III cleaves the DNA backbone mainly through a hydrolytic process (D'Ham et al., 1999). Similarly, posttranscriptional modification of bacterial RNA has been monitored with MALDI-TOF/MS (Kirpekar, Douthwaite, & Roepstorff, 2000). Using this method, the RNA is first digested to completion with a nucleotide-specific Rnase, and the fragments are analyzed by mass spectrometry. A comparison of the masses observed with those predicted from the gene sequence identifies fragments that include modified nucleotides. The authors note that fragments larger than dinucleotides were particularly useful for the identification of posttranscriptional modifications. They also noted that a more complete mapping of RNA modifications would have been obtained with a second Rnase (two Rnases in parallel) combined with fragmentation by post-source decay (PSD). Their approach allowed fast and sensitive screening of purified 5S rRNA from two thermophilic microorganisms, the bacterium *Bacillus stearothermophilus* and the archaeon *Sulfolobus acidocaldarius*, as well as the halophiles archaea *Halobacterium halobium* and *Haloarcula marismortui*. A specific *S. acidocaldarius* posttranscriptional modification was detected and specifically characterized by PSD as a methylation of cytidine₃₂. They reported that the modified cytidine was located in a region that was clearly conserved with respect to sequence and position in *B. stearothermophilus* and *H. halobium*, and to a lesser degree in *H. marismortui*. The extent of dephosphorylation was also monitored in some organisms. Using the same approach, the authors were able to resolve discrepancies within two of the published 5S rRNA sequences.

The potential of IR-MALDI for the analysis of double-stranded DNA was demonstrated in a study by Kirpekar, Douthwaite, & Roepstorff [2000], using restriction enzyme-digested DNA plasmids isolated from *E. coli*. IR-MALDI with glycerol as matrix yielded excellent results for the larger double-stranded DNA after adjustment of the ionic strength via the addition of salts, whereas care was taken to remove salts in the studies mentioned previously. With IR-MALDI, very little fragmentation was observed, and a routine sensitivity in the subpicomole range was obtained for double-stranded analytes with 70 or more base pairs. The authors also noted that denaturation of the double-stranded DNA could be observed with an incremental adjustment of the laser power. Figure 1 shows the MALDI-TOF mass spectrum of a 190 bp double-stranded DNA obtained with laser fluence 1.2 times threshold (a) and at 1.5 times threshold (b). Although the intact double-stranded DNA is detected at about 117 kDa in Figure 1a, only the single-stranded components are observed in Figure 1b, under the influence

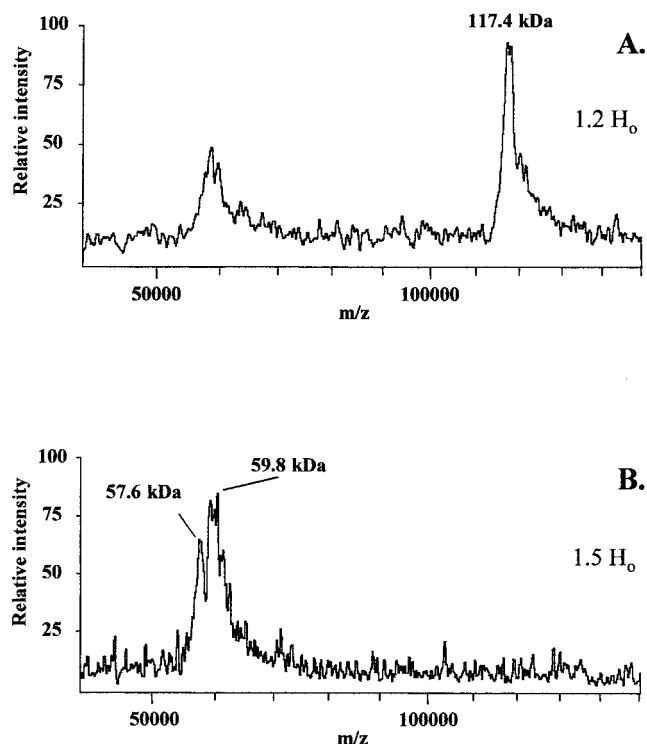


FIGURE 1. (A) The IR-MALDI-TOF mass spectrum of a 0.19 kbp double-stranded DNA obtained with a laser fluence 1.2 times the threshold for detection, and (B) the single-stranded components produced by an increase in laser fluence to 1.5 times the threshold. [Reproduced from Kirpekar, Berkenkamp, & Hillenkamp (1999) with the permission of the American Chemical Society].

of the higher laser power. For smaller DNA fragments, they reported that UV-MALDI with 6-aza-2-thiothymine as the matrix also gave good results. This matrix allowed specific double-stranded complexes that contain relatively few base pairs to be desorbed intact with UV-MALDI. With UV-MALDI, detection of the double-strands was reported up to a 70-mer, but UV-MALDI also produced significant fragmentation, resulting in reduced sensitivity and decreased mass resolution compared to IR-MALDI. Their work clearly demonstrates the advantages of IR-MALDI for sensitive detection of intact bacterial DNA and the fragmentation of DNA that can be observed using UV-MALDI; albeit under UV-MALDI conditions, where ions attributed to intact double-strands are still present.

III. RECOMBINANT PROTEINS

In many of the early MALDI studies of bacteria proteins, the technique was used to characterize recombinant rather than normal host-cell proteins. Studies were done that included the target proteins and unexpected

variants isolated from the host bacteria. The characterization of a set of recombinant proteins that have N-terminal modifications from a target bovine heart fatty acid-binding protein overexpressed in *E. coli* is a typical example (Specht et al., 1994). Similarly, highly purified *E. coli* molecular chaperone Cpn60 (GroEL) was analyzed by MALDI to find a truncated form of GroEL that was verified based on a comparison of gene sequences (Blennow et al., 1995). For more complex problems, affinity-purification techniques have been used to monitor target recombinant proteins isolated directly from whole cells. Parker, Papac, & Tomer (1996) monitored the presence of a recombinant HIV p26 fusion protein expressed in *E. coli* directly from the cells. They detected the enzymatic cleavage of affinity-bound fusion protein and the free fusion protein in solution. They and others have noted that the combination of mass resolution, sensitivity, and speed of analysis made MALDI-TOF mass spectrometry an attractive alternative to SDS-PAGE or gel electrophoresis in these and similar applications. The validity of their claim was well-supported in a careful comparison of the methods (Easterling et al., 1999). This study was done for proteins with molecular masses that ranged from 5 to 50 kDa. In this comparison, MALDI-TOF/MS was coupled with a rapid sample preparation step that allowed the analysis of cells directly from the growth media with a total time for analysis that was reported to be less than 10 min. In side-by-side comparisons using criteria such as speed, efficiency, resolution, and mass assignment accuracy, MALDI-TOF/MS showed clear advantages. More important than these criteria, however, was the fact that the mass spectrometry approach was also able to identify the chemical nature of posttranslational modifications and other deviations from the expected chemical structure, which often go unrecognized with chromatographic approaches.

Figure 2 shows data from one of these experiments, and demonstrates the rapid and simple detection of such modifications with MALDI. In this case, the rapidity of the method is sufficient to allow temporal-based changes to be detected in a dynamic process. The MALDI-TOF mass spectrum obtained 3 h after induction of the protein PfRd is shown in the Figure 2. As the figure shows, the targeted protein (PfRd) is much less abundant than the methionine (+Met) and formylated methionine (+Met_f) attached species. The overexpressed proteins were sufficiently abundant that they dominated the mass spectrum, even when detected directly from the whole cells used in this study. As noted above, because of the rapidity of the analysis from whole cells rather than a complex isolation procedure, it was possible to monitor the relative abundances of several proteins as a function of time after induction so as to optimize the production of the desired product. Similar rapid detection of expressed

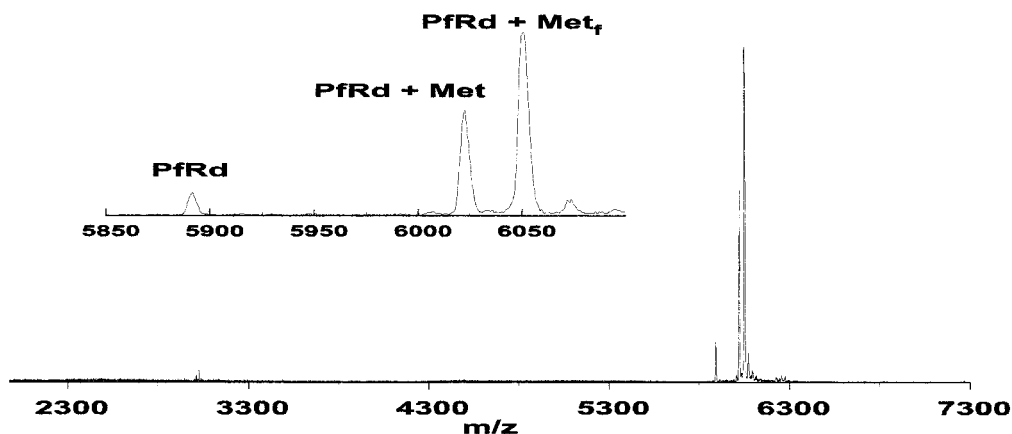


FIGURE 2. MALDI-TOF mass spectrum of *E. coli* cells used for PfRd expression obtained 3 h after induction. The inset shows that the unwanted methionine (PfRd + Met) and formylated methionine (PfRd + Met_f) variants of this protein dominate under these experimental conditions. [Reproduced from Easterling et al. (1998) with the permission of the American Chemical Society].

proteins could be monitored as a function of other changes as other fermentation conditions are varied. These authors also reported that the analysis of higher molecular weight proteins was enhanced when the bacterial solutions are first sonicated. (The detection of proteins directly from cells as well as the detection of higher molecular weight proteins is discussed in subsequent sections below). Many other recombinant proteins isolated from *E. coli* have been characterized with MALDI. Several examples of such studies may be found (Zanette et al., 1998; Franzen et al., 1999; Fucini et al., 1999; Jin, Sun, & Chitnis, 1999; Jungblut, Grabher, & Stoffler, 1999; Leppanen et al., 1999; Yan et al., 1999).

IV. PROTEIN IDENTIFICATION

The analysis of naturally occurring proteins in bacteria is a logical extension of the analysis of overexpressed recombinant proteins. The experiments may be more difficult because of the smaller amounts of protein present or because the identity of the target protein is not known in advance. Nevertheless, the MALDI methodology for naturally occurring bacterial proteins need not be different than the approaches applied to detection of recombinant proteins in bacterial cells. As was done in the studies of recombinant proteins, the usual approach has been the analysis of proteins isolated from bacteria after a preliminary isolation step that produces one or more fractions that contain the target proteins. The characterization of examples from two atypical classes of bacterial proteins (metalloproteins and desulfurization proteins) typifies the approach. Two copper proteins, azurin-1 and azurin-2, isolated from

Alcaligenes xylosoxidans GIFU1051, a denitrifying bacterium, were characterized with MALDI (Fukuo et al., 1998). From an acidified matrix (2,5-dihydroxybenzoic acid), the proteins were detected as positive ions without any evidence of a copper atom. The molecular weight for azurin-1 was found to be 14,017.6, within 2 Da of the value calculated from the presumed amino acid composition based on the same protein in other bacteria. However, the spectrum of the azurin-2 protein isolated from *A. xylosoxidans* indicated a molecular weight of 13,748.2. This value was about 26 Da lower than the value that was expected (13,774.6 Da). Moreover, the molecular weight determined by electrospray MS was 13,740 Da. Based on both molecular weight determinations, the mass difference was attributed by these authors to a single different amino acid residue (Met to Val) compared to a known azurin-2 protein isolated from another organism, *A. xylosoxidans* NCIB11015. It is not clear how, on any chemical basis, these authors ruled out any of the other single amino acid changes (for example C to A, H to C, W to N, etc.), which also correspond to a mass difference of from 26 to 35 Da. The fact that their azurin-2 differed from an already known form was easily established based on molecular weights obtained using either MS method because both measured mass values were significantly less than the expected value. However, any assignment of specific amino acid differences that lead to these two variant forms of the azurin-2 protein would seem realistically to require complementary data. These data could include tandem MS data, accurate mass assignments, or other information that would justify assignment of a specific single change in the amino acid composition that gives rise to the observed mass difference. The use of exact

mass values and protein mapping for characterizing similar amino acid differences was demonstrated in the other study. It involved bacterial enzymes being developed for use in petroleum desulfurization. For a specific enzyme, *dsz-C*, from *Rhodococcus sp* strain IGTS8, a prior discrepancy in residue assignments was resolved, using proteolytic digests and MALDI-TOF/MS (Wolf et al., 1998). Using delayed extraction, ion reflection, and mass accuracies of better than 40 ppm, MALDI mass spectral data were used to establish a single correct sequence for *dsz-C*, resolving a discrepancy that resulted from a posttranslational loss of an N-terminal methionine.

MALDI-TOF mass spectrometry has been used to investigate unknown proteins isolated from bacteria and mollicutes. *Spiroplasma melliferum* is a simple wall-less, helical bacterium from which proteins have been isolated and characterized by MALDI. This organism has a genome of approximately 1460 kbp that encode only 800–1000 gene-products. In one study, amino acid analysis and MALDI-TOF mass spectrometry were used to identify 9 of 12 proteins isolated from this organism, which was otherwise poorly defined at the molecular level (Cordwell et al., 1995). In a similar study, 19 proteins from the smallest known self-replicating organism, *Mycoplasma genitalium* were subjected to peptide-mass fingerprinting MALDI-TOF mass spectrometry (Wasinger et al., 1995). Although the majority of the proteins from both organisms were not characterized, the combined approach of amino acid analysis and peptide-mass fingerprinting with MALDI allowed gene products to be linked to homologous genes in a variety of organisms. A more complete characterization of the proteome of *S. melliferum* was demonstrated in a follow up-study (Cordwell, Basseal, & Humphery-Smith, 1997). In this study, a two-dimensional electrophoresis gel reference map showed 456 silver-stained and replicated protein spots, of which 156 were further characterized using one or some combination of the following: amino acid analysis, peptide-mass fingerprinting via MALDI-TOF mass spectrometry, and N-terminal protein microsequencing. Once again, several proteins with a close relationship to those previously determined from other species were identified. Other proteins from this organism were either reported as hypothetical, or having no known close relative. Gene-products from several major families involved in activities such as glycolysis, translation, transcription, cellular processes, energy metabolism, and protein synthesis were identified in a study of the organism's proteome. Interestingly, several gene-products characterized in *S. melliferum* were not found in the entire *M. genitalium* and *Mycoplasma pneumoniae* (closely related Mollicutes) genomes.

Proteins associated with the application of environmental stress to bacteria have been investigated with

MALDI-TOF mass spectrometry. A protocol that included 2D SDS-PAGE, electroblotting onto nitrocellulose membranes, CNBr cleavage, MALDI-TOF/MS, and a database search, was developed for the identification of proteins associated specifically with the response of *E. coli* to hypochlorous acid stress (Dukan et al., 1998). MALDI analysis of proteins and CNBr fragments was accomplished directly by dissolving the nitrocellulose membrane in an acetone solution of the matrix. This step eliminated the need for recovery of the protein from the membrane. Spectra were readily obtained from picomole quantities of proteins. Nineteen proteins that seemed to exhibit a response to hypochlorous acid stress were either confirmed or identified on the basis of this study. Figure 3 shows the MALDI-TOF mass spectrum obtained from one of the nitrocellulose membrane spots after CNBr cleavage. In addition to the singly and doubly charged protein (signals at 37.1 and 18.6 kDa) observed from untreated spots, several unique CNBr fragments are identified on the figure. Although the combination of molecular weights from the parent protein and the masses from the CNBr digest fragments allowed an unambiguous identification of some spots, others were not identified. Three spots showed no agreement with fragments predicted from the *E. coli* genome, whereas four others showed no CNBr-induced fragments. The authors suggested that this difference might be because they did not contain a methionine residue.

Environmentally induced proteins have been monitored by MALDI after the use of other analytical approaches for the preliminary protein isolation steps. For example, the *E. coli* proteins responsive of to L-arabinose induction were detected and identified, using a combination of non-porous packing reversed phase separations of the water-soluble proteins obtained from whole cell lysates (Wall, Lubman, & Flynn, 1999). Likewise, other kinds of proteins have been detected in studies that involved the direct detection from a separation medium. Intact *E. coli* proteins separated by isoelectric focusing-immobilized pH gradient (IEF-IPG) gel separation were analyzed by MALDI directly from the gels (Loo et al., 1999). In this study, which compared MALDI with ESI, both methods showed sub-picomole sensitivity and good mass measurement accuracy for the *E. coli* proteins. However, the use of an array detector was necessary for the ESI-MS analysis to discriminate against contaminating background ions and to selectively detect the intact high-mass protein ions. Moreover, MALDI demonstrated a higher throughput for the analysis of proteins from the gels. The authors noted the conceptual similarity between their approach and the traditional approach using a 2D-gel, and coined the term "virtual 2-D" gel for their method-with a first-dimensional IEF separation and the second dimensional determination by MALDI-TOF/MS.

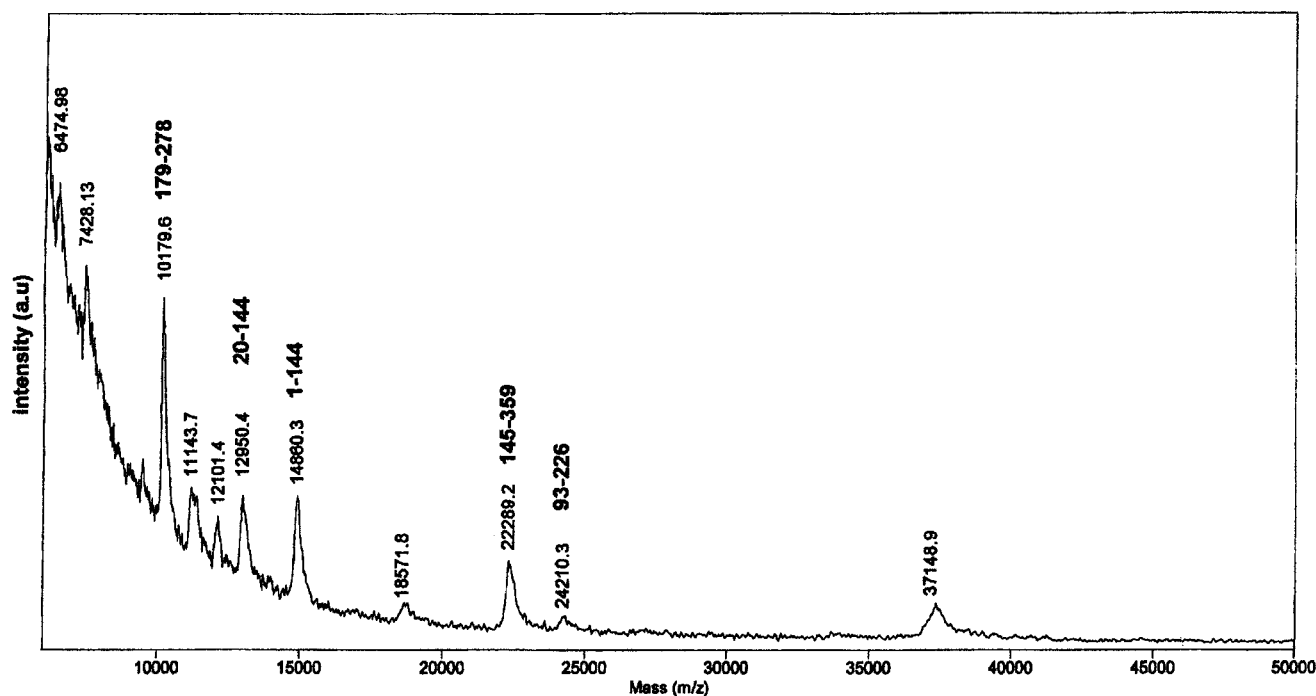


FIGURE 3. MALDI-TOF mass spectrum of a nitrocellulose spot after CNBr cleavage that shows measured masses and postulated protein fragments. [Reproduced from Dukan et al. (1998) with the permission of the American Chemical Society].

MALDI-TOF/MS played an important role in characterizing the proteins of the gastric pathogen, *Helicobacter pylori* (McAtee, Fry, & Berg, 1998). Gel electrophoresis was used in combination with Western blotting to separate and identify potential antigens of *H. pylori* strain Z-170. Proteins found to be reactive with pooled sera from 14 infected patients were individually digested in situ, and the resulting fragments were analyzed with MALDI-TOF/MS. The mass spectral data were compared with predictions from the *H. pylori* genome DNA sequence, and on this basis 20 proteins were identified. This “proteome” approach for the identification of previously unknown proteins will be useful for examining regulation of gene expression and protein localization, and for the development of serologic tests to detect human infection.

V. PROTEOMICS

Some of the studies listed in the previous section could be considered to be proteomics studies, and several authors described their studies that way. Applications that involve proteomics generally require the detection of the largest possible number of proteins. For MALDI, this goal

generally requires the analysis of isolated proteins or many protein-containing fractions. Several of the studies described above used gels to produce large numbers of proteins or protein containing spots. Clearly, the analysis of isolated proteins requires additional steps compared to the simpler analyses of cell lysates, cell extracts, or whole bacteria described later. Although these additional steps preclude the “rapid analysis” possible using whole cells, the analysis of isolated proteins offers an important advantage critical to proteomics applications. The number of proteins detected from bacterial fractions is larger than the number that would be detected from a single sample or whole cells. As a rule, the number of proteins that can be detected by MALDI increases with the number of fractions collected. This factor was amply demonstrated when high performance liquid chromatography (HPLC) was used to separate components in a solvent suspension of *E. coli* prior to off-line MALDI-TOF/MS analysis of the collected fractions (Dai, Roser, & Long, 1999). When the number of peaks found in each fraction was considered, this experiment resulted in the detection of over 300 peaks in the 2–19 kDa mass range. This number represented an order of magnitude increase over the number of components observed with direct MALDI analysis of the same proteins in a mixture. In this

same study, several of the HPLC fractions were subjected to MALDI analysis after proteolytic digestion. This step led to the identification of three components as specific proteins that are normally expected to be present in *E. coli*. Figure 4a shows the MALDI-TOF mass spectrum from an HPLC fraction from *E. coli*, whereas Figure 4b shows a tryptic digest of the same fraction. Based on the protonated molecule ion at m/z 7273 and the masses of the tryptic fragments (see also Table 1), the protein was identified as *E. coli* CSP-C.

Whereas chromatographic or other separation steps can increase the total number of proteins that can be detected from bacteria by MALDI, it does not eliminate all of the problems with preferential representation of some proteins over others in the spectra. This difference was demonstrated in a surprising study where the authors

TABLE 1. Matched peptide fragments from the tryptic digestion of fraction #65 in Figure 4

Tryptic fragments	Protonated molecule ion $[M + H]^+$	
	Theoretical fragments	Identified fragments
9–14	810.4	810.2
1–8	871.6	871.2
56–68	912.5	912.5
15–26	1196.6	1196.7
5–14	1222.6	1222.8
27–41	1666.9*	1666.7*
42–58	1907.1*	1906.8*
42–68	2801.1*	2801.7*
15–41	2845.2*	2845.8*

Data taken from Dai et al. (1999). *Average mass of the protonated molecule ion.

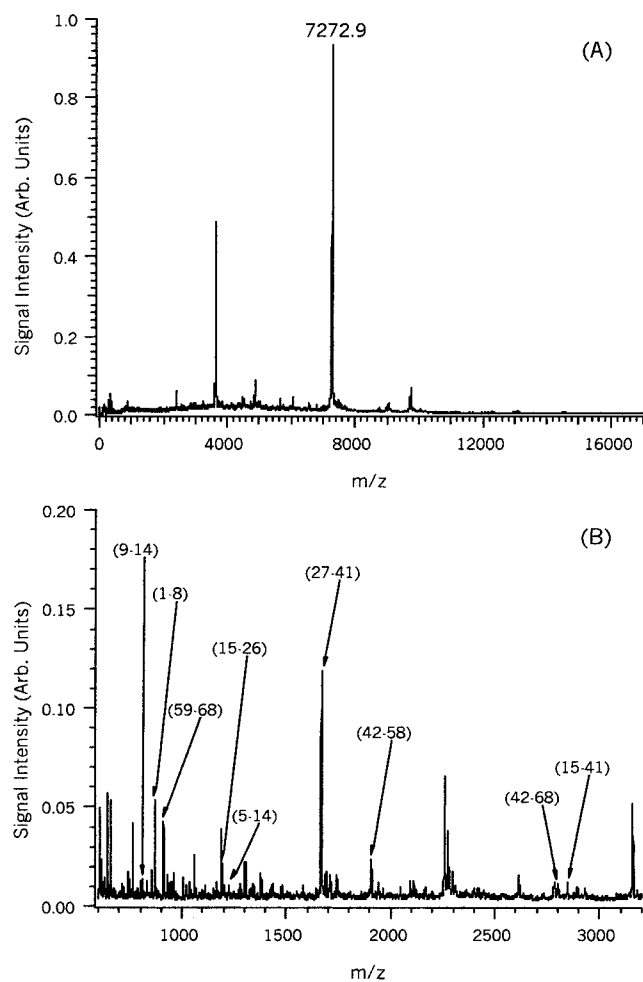


FIGURE 4. MALDI-TOF mass spectra from a protein-containing fraction isolated from *E. coli* (A) before, and (B) after tryptic digestion. [Reproduced from reference Dai et al. (1999) with the permission of John Wiley].

noted that bacterial proteins from in-gel digests were not equally represented in the IR-MALDI-TOF mass spectra. In the course of a study of over 100 mycobacteria proteins produced by tryptic in-gel digestion and 2D gel separation, they found that 94% of the most intense MALDI-MS peaks were for peptides that have arginine rather than lysine at the C-terminal end (Krause, Wenschuh, & Jungblut, 1999). The expected ratio would be more like 50%. This phenomenon was reproduced from an equimolar mixture of the synthetic peptides known to be present in the tryptic digest of the mycobacterial 35 kDa antigen, and again from several binary mixtures of synthetic peptides that differ only at the C terminus (Arg or Lys). That this result was a MALDI rather than a biochemistry-related phenomena was confirmed based on the HPLC and the electrospray MS responses. Specifically, the protein mixtures showed responses quite different by HPLC or ESI than by MALDI with respect to relative abundance, and the intensity values were more representative of the expected proportions of the two types of proteins. This result was easily confirmed by the analysis of the mixtures of synthetic peptides that have known compositions. The differences between data from MALDI and ESI analysis of the known samples (which gave data more representative of the correct molar ratios) is indicative of a complex phenomenon. In additional studies, they determined that the extent of the preferential observation of C-terminal arginine-over C-terminal lysine-containing peptides was dependent upon the MALDI matrix used.

The known genome of *H. pylori*, a pathogenic organism associated with gastritis, ulcer, and stomach carcinoma, which infects nearly half of the world's population, was used in conjunction with MALDI-TOF/

MS in a study of the functional proteome of this organism (Jungblut et al., 2000). From 1800 protein types, peptide mass fingerprinting was used to identify 152 proteins, including 9 known virulence factors and 28 antigens. Interestingly, the three strains investigated had only a few proteins in common. This finding explains why taxonomic identification has been possible with MALDI even when only a few proteins present in the cell are detected in the simplest MALDI experiments described later. In the study in Jungblut et al. (2000), the expression of 27 predicted conserved hypothetical open reading frames (ORFs) and six unknown ORFs were confirmed. As noted in other studies, the growth conditions for bacteria also had an important effect on the presence of certain proteins, and this factor also had to be taken into account.

VI. OTHER APPLICATIONS THAT INVOLVE CELLULAR ISOLATES

MALDI-TOF/MS has also been used to study the non-covalent association of bacterial proteins. One such study involved the associations that involve proteins that correspond to virulence factors isolated from bacteria (Moniatte et al., 1996). Aerolysin, a virulence factor secreted by *Aeromonas hydrophila*, is typical of a group of beta-sheet toxins that must form stable homoooligomers to generate channels into biological membranes that allow their insertion into host cell membrane-tissue. MALDI-TOF mass spectrometry was used to measure the mass of the isolated aerolysin oligomer. A mass of 333,850 Da was measured, in reasonable agreement with a heptameric complex with an expected mass of 332,300 Da. These results confirmed earlier electron microscopic and other evidence that the aerolysin oligomer existed as a heptamer. This example demonstrates the use of MALDI-TOF mass spectrometry as a tool to study or confirm a typical, yet biologically important, non-covalent association found in bacterial proteins.

MALDI-TOF/MS has been used to study bacterial metabolism. One such study monitored the metabolic changes associated with the growth of cells from only selected carbohydrates. When *Streptococcus oralis* was cultured with ribonuclease B as the only source of carbohydrate, a selective use of the sugars of the Man5 glycoform was observed (Tarelli et al., 1998). This result was attributed to novel alpha-(1->3), alpha-(1->6) and beta-(1->4) mannosidase activities that acted in a concerted manner in a single-step process. This selective use of Man5 was explained by the absence of an alpha-(1->2) mannosidase in the bacterium, which is required to initiate the breakdown of the glycan chains present in the other glycoforms.

VII. WHOLE CELLS

The taxonomic identification of bacteria based on constituent proteins represents a logical extension of the methods developed for bacterial proteomics studies. Ideally, this approach should be rapid and yet be based on a sufficiently large group of proteins so that a unique mass spectral fingerprint for the organism or strain can be obtained. Lubman's group demonstrated that MALDI-TOF mass spectrometry could be used to obtain characteristic profiles from the proteins isolated from disrupted cells. MALDI-TOF/MS of protein-containing isolates was suitable for identification of bacteria (Cain, Lubman, & Webber, 1994; Liang et al., 1996). They simply harvested cells by centrifugation, washed them with buffer, resuspended the bacteria, and disrupted them with sonication. Finally, a protein-containing fraction was collected from a methanol precipitate for the final MALDI-TOF analysis. Alternatively, the proteins were also separated by capillary liquid chromatography and collected for off-line analysis by MALDI-MS (Liang et al., 1996). Using this second approach, bacteria have been discriminated based upon their overall protein profiles to the species level, from only pmol levels of protein. The use of magnetic beads and avidin-biotin technology has also been proposed as a means of recovering biotinylated bacterial proteins directly from cells (Girault et al., 1996). The elution of peptides from the beads is achieved by mixing the beads with the MALDI matrix solution and removing, after a few minutes, the beads with a magnet. The matrix solution that contains any biotinylated peptides is directly mass analyzed by MALDI. Using either approach, the proteins can be pooled prior to MALDI analysis to give a characteristic fingerprint.

Holland et al. (1996) proposed a similar approach, using the protein profile detected directly from whole cells rather than cellular extracts. To demonstrate the methodology, blind-coded samples of whole-cell bacteria were identified either by comparison with archived reference spectra or based on co-analysis of bacteria from reference cultures. This method was extremely rapid. Bacteria were sampled from colonies on an agar plate, mixed with the matrix, air-dried, and introduced in batches into the mass spectrometer for analysis. In all of the spectra obtained in these experiments, each bacterial strain showed a few characteristic high-mass ions that were attributed to bacterial proteins. Although this and similar approaches only detect that fraction of proteins that can be easily sampled from the whole cells in a complex environment, the profiles are, nevertheless, generally suitable for taxonomic identification. This suitability arises because the biological variation in the proteins present at the strain level is more than sufficient to allow rapid identification with this approach, even though only a

fraction of the proteins present are sampled. Similar results were reported independently by Claydon *et al.* at about the same time (Claydon *et al.*, 1996). They reported an automated analysis that produced genus- and species-specific spectra from cells removed from colonies within minutes. In addition to identification of microorganisms from different genera and different species, they also reported different spectra from strains of the same species. Their procedure provided a unique mass spectral fingerprint of the microorganism from subcultures grown for 3-day and 6-day periods, and from the same cultures 1 day later, as well as from fresh subcultures after 2 months. They attributed the MALDI-TOF mass spectral signals to desorbed components of the cell wall. Both of these studies were confirmed by a third study that appeared shortly thereafter (Krishnamurthy & Ross, 1996), which included bacteria from pathogenic and non-pathogenic strains.

Some of the difficulties involved in the MALDI-TOF mass spectral analysis of bacteria have been the complexity of the spectra, the large mass ranges used, and the subtle differences that may be observed in spectra from related stains. Figure 5 shows spectra obtained from four strains of *E. coli* by Arnold and Reilly (1998). The spectra shown in this figure are typical of “whole cell” or “intact cell” spectra obtained in many laboratories that show a large number of peaks between m/z 3000 and 10,000. Although other spectra may contain higher-mass ions, a significant part of the MALDI spectral “fingerprint” from bacteria typically occurs within this mass range, even in experiments where higher-mass ions are also observed. These authors also reported that their spectra were typical of the sort of spectral differences observed in all of their strains. As the figure shows, these *E. coli* strains share many peaks in common, but also have some unique ions as well. For example ions near 3630, 3850, 4170, 4780, 5100, 5380, 7280, 8320, 9070, 9530, and 9740 Da seem to differentiate one or more strains from the others. However, the spectra from replicate samples of the same strain can also show spectral differences. To measure the strength of apparent spectral differences, this group developed a mathematical approach to compare MALDI-TOF mass spectra from whole bacteria, using replicate spectra. This mathematical fingerprint-matching technique eliminated the need for subjectivity in comparing spectra visually to determine whether or not they matched or were different. Using this approach, Arnold and Reilly (1998) readily distinguished 25 different strains of *E. coli*. Cells were grown in culture, samples were prepared, and MALDI-TOF mass spectra were recorded in a manner comparable to the previously reported studies. Pairs of spectra were compared based on their method, a modified cross-correlation procedure. This approach increased the sensitivity of the important small spectral differences that often

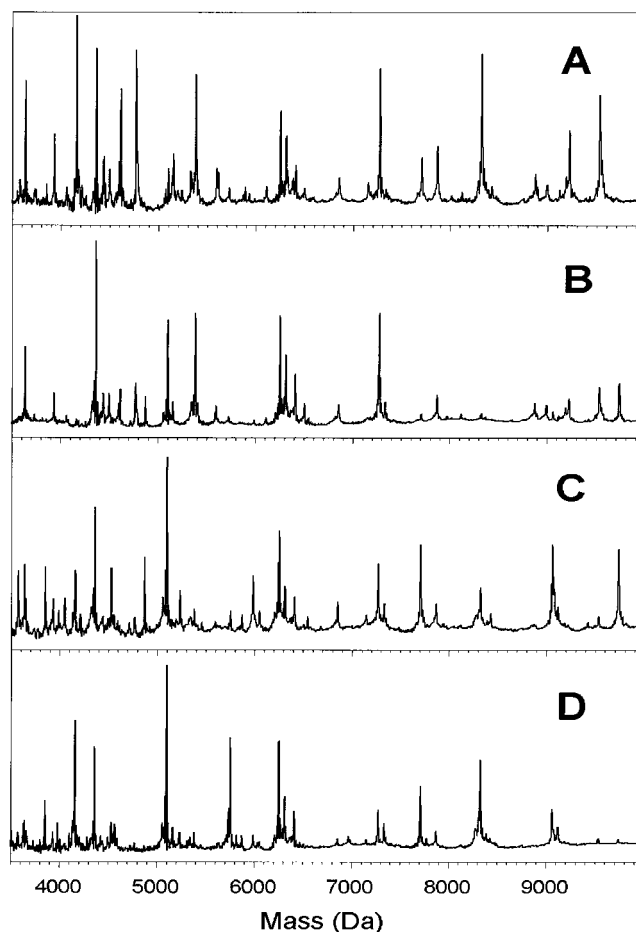


FIGURE 5. MALDI-TOF mass spectra from cellular suspensions of four strains of *E. coli*, (A) BLR, (B) XL1 blue, (C) RZ1032, and (D) CSH23 that show the typical “whole cell” pattern of peaks from m/z 3000 to 10,000. [Reproduced from Arnold & Reilly (1998) with the permission of John Wiley].

allow strains to be distinguished. These authors also reported that their technique is adjustable, and can be “fine-tuned” to facilitate its use in other studies with bacteria that have more or less similar spectral patterns. This goal is accomplished by simple adjustments of the parameters used in the cross-correlation procedure.

The successful use of comparisons of spectral patterns for strain differentiation has to be at least somewhat dependent upon the reproducibility of spectra. However, reproducibility has been a difficult problem in experiments that involve MALDI of cells, and large variations have been seen in spectra obtained under different conditions. There are many experimental parameters that can have a strong effect on the observed mass spectra. Wang *et al.* (1998) addressed the mass spectral reproducibility issue experimentally in a careful interlaboratory

experiment where the effects of a number of parameters were systematically investigated. In that work, they demonstrated that minor variations in the sample/matrix preparation procedures for MALDI and in the experimental conditions used for bacterial protein extraction or analysis could result in significant changes in the resulting spectra. However, they also noted that a subset of peaks was conserved in spectra obtained even under quite different experimental conditions so long as spectra were from genetically identical bacteria. These conserved peaks helped explain the successful application of MALDI-TOF/MS to bacterial characterization even prior to the standardization of experimental conditions. They and others have proposed that these specific selective (conserved) biomarker proteins could be used for bacterial identification irrespective of changes in other biomarkers.

Another initially perplexing issue was the time dependence of spectra noted upon analysis of bacteria from cultures (Arnold et al., 1999; Lay & Holland, 2000). Whereas changes in bacterial spectra over time complicated pattern recognition approaches, biologically based changes in the protein composition of cells might have been anticipated. Bacteria respond rapidly to environmental changes, and the production of stress proteins or other similar changes in cellular processes when cells are stored, handled, or cultured over different time periods prior to analysis results in non-identical samples for reasons associated with the biology of bacteria. Arnold et al. (1999) reported the time evolution of MALDI spectra taken from a growing bacterial culture by periodically removing and analyzing whole cells. Their mass spectra contained tens of peaks in the 3–11 kDa mass range. As shown in Figure 6, when cultures of *E. coli* strain K-12 were grown under otherwise identical conditions and sampled periodically from 6 to 84 h after inoculation, the spectra varied considerably in the numbers and intensities of peaks. In several experiments, they observed that the relative intensities of several of the stronger peaks changed dramatically and consistently over time. They correctly noted that such temporal characteristics must be taken into account when MALDI-TOF/MS is applied to identify bacteria, and that bacterial incubation time is one of the variables that should be carefully controlled in experiments aimed at bacterial identification. Their results also demonstrated that MALDI-TOF/MS of whole cells could be used to monitor biological changes, such as those that occur during the aging of a bacteria colony. This whole-cell approach to the monitoring of biological changes would, of course, only allow a fraction of the environmental-response related proteins to be detected, compared to similar studies with isolated or fractionated protein samples, but the rapidity of the analysis often offsets this limitation.

In another study that involved longer time periods, Welham et al. (1998), working with a variety of Gram-positive and Gram-negative bacteria, reported that spectral changes were observed based on the passage of time and the amount of loaded sample. Nevertheless, even with 3 months between experiments, spectra contained a number of relatively reproducible ions. This work provides additional evidence on the existence of conserved identity-specific biomarker ions from MALDI-TOF mass spectra of cells. It is also noteworthy that, although most studies have detected proteins below 20 kDa, this group reported the detection of some proteins with masses approaching 40 kDa. Figure 7 shows one of their spectra from *E. coli*. Although the bulk of the signal is below 20 kDa, the spectrum does show weaker signals that correspond to higher molecular weight proteins, especially the signal near 34 kDa. A number of high-mass ions were also noted in a study by Winkler, Uher, & Cepa, 1999, who reported that differentiation of *Campylobacter jejuni*, *Campylobacter fetus*, *Campylobacter coli*, *H. pylori*, and *Helicobacter mustelae* was facilitated by the presence of ions that ranged up to 62,000 Da. In this study, a biomarker for *H. pylori* was centered on m/z 58,268, whereas *H. mustelae* was distinguished from *H. pylori* based on ions at m/z 49,608 and 57,231. It is not yet clearly understood the extent to which these high-mass ions can be attributed specifically to these organisms, which are all related. The possibility remains that perhaps subtle differences in the methodology used for the measurements might have affected the detection of ions at these higher masses. It is interesting to note that, in a 1999 study by Winkler, Uher, & Cepa, an analysis time dependent behavior with respect to the detection of high-mass ions was noted. The bacterial cultures were suspended in 50% methanol (or 0.1% TFA) prior to analysis. The length of time between the initial suspension of the bacteria and analysis with MALDI effected the detection of high mass ions, which showed significant differences (decreases in intensity) within time periods as short as 1 h. This observation suggested that perhaps more high-mass ions might be observed when methods have been optimized to facilitate their detection.

Madonna et al. (2000) have studied methods to facilitate the detection of high-mass ions. They recently reported results from a study of the factors that might facilitate the detection of high-mass ions from bacteria, including the detection of high-mass ions for Gram-positive and Gram-negative bacteria, using a ferulic acid (FA) matrix. Their matrix consisted of 12.5 mg of FA dissolved in 1 ml of a solution with a composition of 17% formic acid, 33% acetonitrile, and 50% water. The procedure involved depositing growing bacteria colonies from culture dishes directly onto the MALDI probe, and treatment first with 40% ethanol and the FA matrix

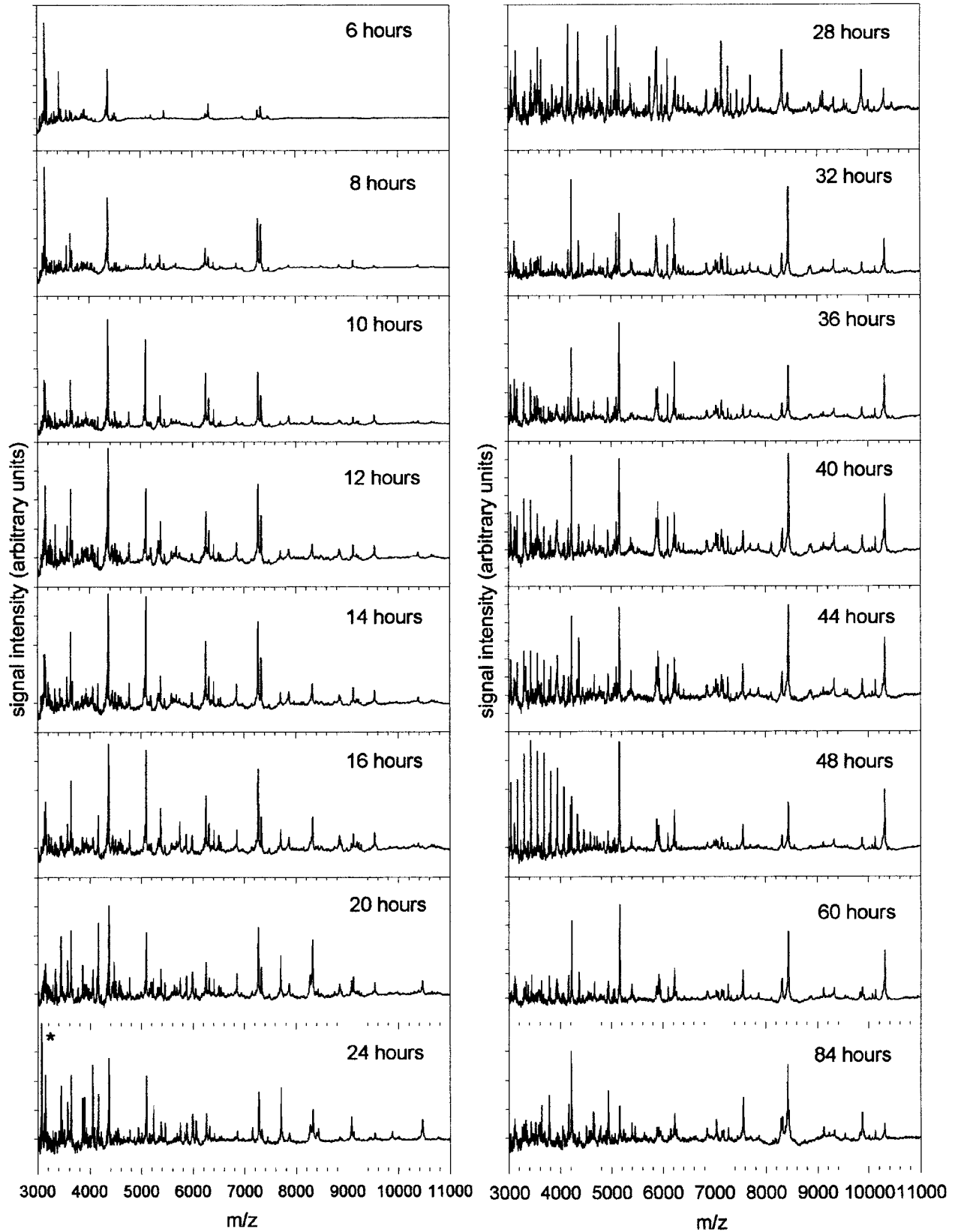


FIGURE 6. MALDI-TOF mass spectra from *E. coli* K-12 sampled 6-84 h after inoculation. [Reproduced from Arnold et al. (1999) with the permission of the American Chemical Society].

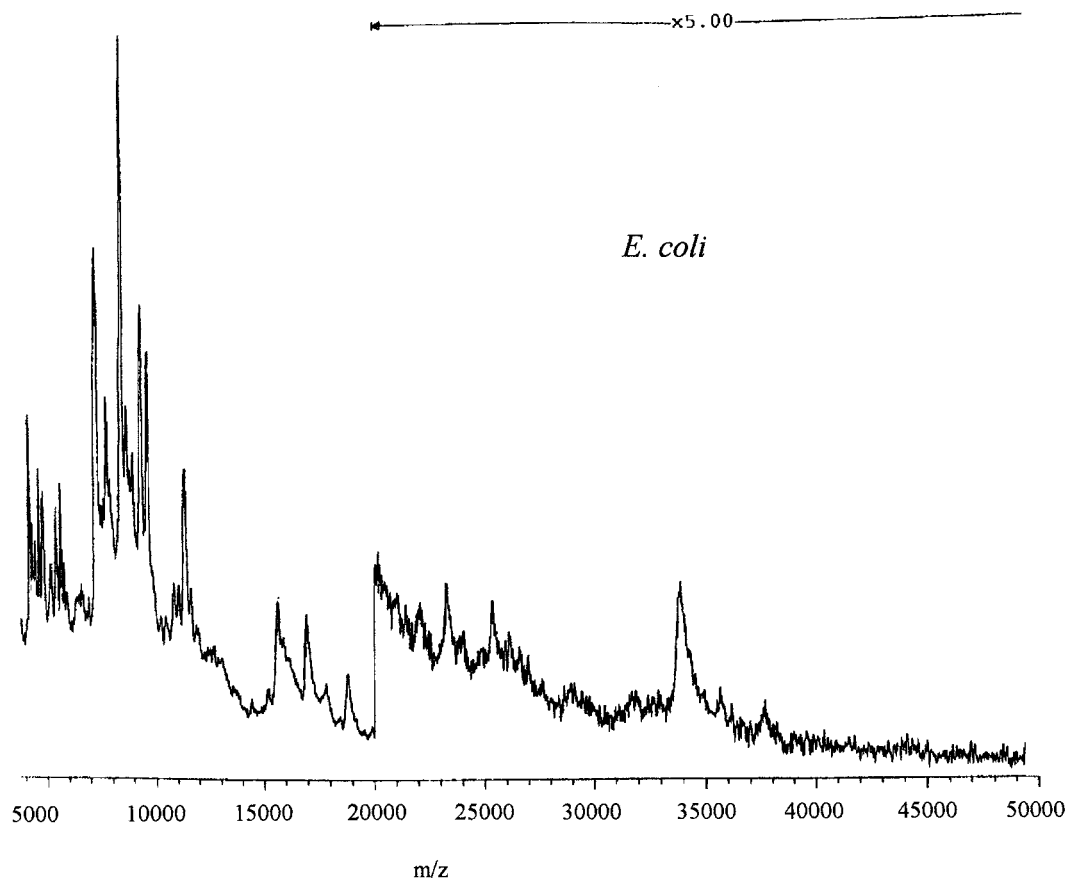


FIGURE 7. MALDI-TOF mass spectrum from an *E. coli* strain obtained after vortexing cells for 30 sec in 2,4-hydroxyphenylazobenzoic acid matrix. [Reproduced from Welham et al. (1998) with the permission of John Wiley].

solution described above. Using this approach, high-mass ions were observed along with a substantial number of intense signals in the more ‘conventional’ fingerprint region that extended to 20 kDa. Like Winkler, Uher, & Cepa, 1999, they suggested that high-mass ions might be particularly important to the differentiation of some bacteria based on the rarity of high-mass signals and the minimal background present in the higher mass region of bacterial MALDI spectra. They illustrated the importance of using high-mass ions for bacterial differentiation in an interesting experiment that involved a mixed culture containing *E. coli* and *Proteus mirabilis*. The high-mass portion of the mass spectrum from this mixed culture is shown in Figure 8. Bacteria-specific ions for the two organisms are marked with different symbols. It is clear that at least six characteristic ions for each organism are observed between 15 and 70 kDa. This experiment also demonstrates the fact that, at least for this mixed culture, the presence of one organism does not suppress the formation of ions from another organism. As the authors point out, the

ability to detect ions from coexisting bacteria in mixtures will play an important role as MALDI-TOF/MS is extended to many potential “real-world” applications.

Despite the evidence presented above that FA might be an optimum matrix for MALDI-TOF mass spectrometry of whole cells, more experiments need to be done. A review of the larger body of evidence regarding the matrix compounds recommended (or simply used) by various investigators in their own studies suggests that instrument-related parameters significantly impact upon the choice of matrix that should be used. The type of laser and the identity of the matrix compound used (or the optimum compound if more than one was used) as reported in 27 of the works cited in this review are summarized in Table 2. Except for a few studies that involved the analysis of spores, the use of corona plasma discharge (CPD) or the analysis of purified proteins, the majority of the studies listed in this table involved the analysis of whole cells by UV-MALDI with either a nitrogen or an Nd:YAG laser. For those experiments with a nitrogen

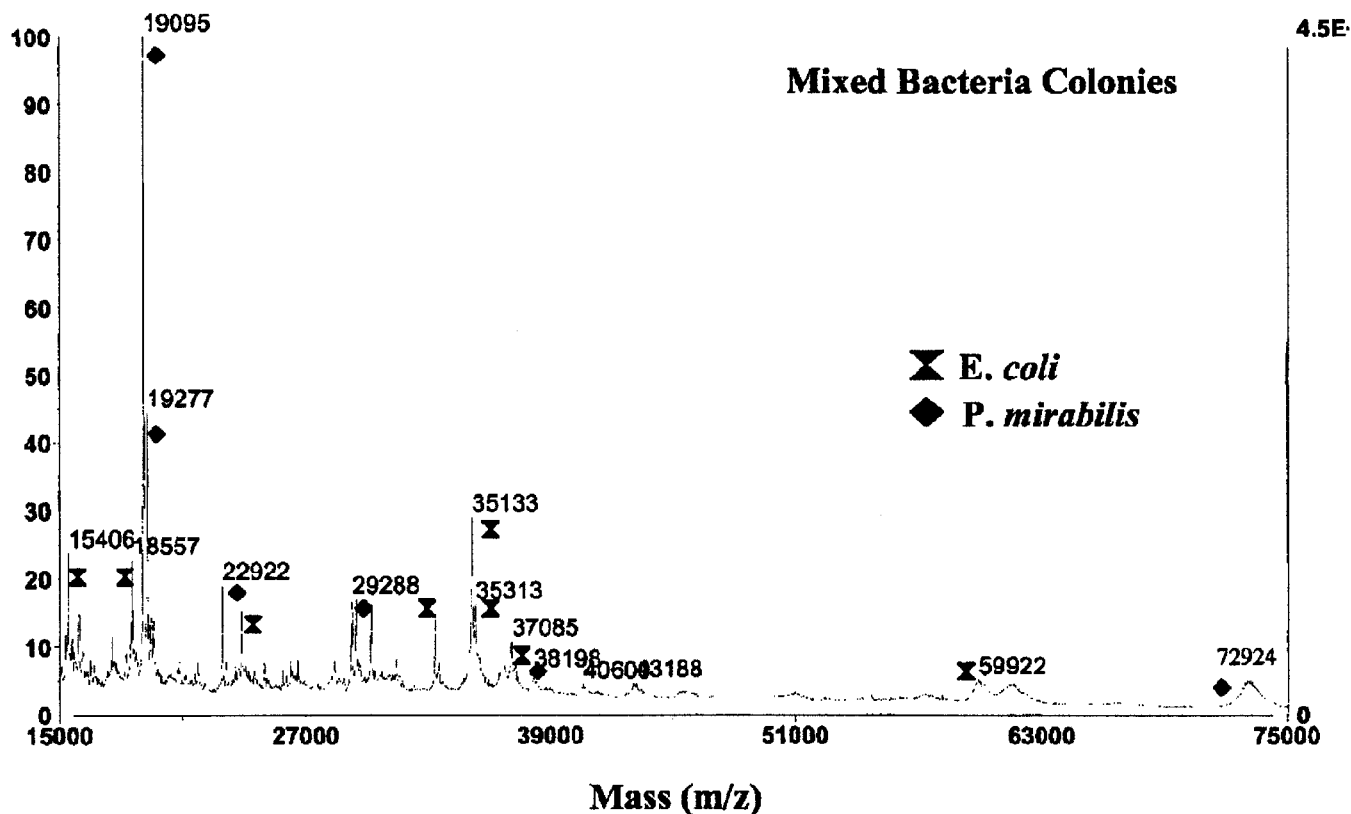


FIGURE 8. MALDI-TOF mass spectrum from a mixture of *E. coli* and *P. mirabilis* obtained with FA as the matrix. [Reproduced from Madonna et al. (2000) with the permission of John Wiley].

laser, five different compounds were reported to be either the best or only compound used as the MALDI matrix. These five compounds were sinapinic acid (SA), α -cyano-4-hydroxycinnamic acid (HCCA), 2,4-hydroxyphenyl benzoic acid (HPBA), 2,5-dihydroxybenzoic acid (DHBA), and FA. In light of the conflicting data, even from studies that involved direct comparisons of matrix compounds, the optimum matrix compound for use with the nitrogen laser remains to be conclusively determined, although the high-mass ions observed in some studies with FA has generated much interest (Madonna et al., 2000). On the other hand, for all of the studies listed in Table 2 where a Nd:YAG laser was employed, HCCA was reported as the best or only matrix compound. Because several of these studies also involved direct comparisons of HCCA with other matrix compounds, it can be inferred that the optimum matrix for studies with the Nd:YAG laser is HCCA. This result is especially true because sufficient time has elapsed since the introduction of FA as a matrix compound for the appropriate studies to have been completed, and yet no conclusive reports of high mass ions from the FA with an Nd:YAG laser have yet been reported.

Another study reported an unusual sort of time-related response. Significant time-dependence behavior was observed for spectra from one bacterium, but not another (Saenz et al., 1999). *E. coli* and *Bacillus atrophaeus* spectra were collected on 10 different occasions over a 3-month period by two different operators. As expected, these analyses resulted in the detection of biomarkers in the m/z 2,000–20,000 range, including some peaks reported previously. However, although the *E. coli* spectra changed significantly over time, the spectra from *B. atrophaeus* changed very little. The authors of this study noted that changes in the person who operated the instrument might have played a role in the apparent time-related changes in the spectra; however, the intriguing possibility that this simple observation might reflect profound biological differences between these two very different bacteria remains to be investigated more completely.

Despite the large number of variations in the “whole cell” MALDI methods that have been tested, the determination of an “optimum” set of conditions for the production of characteristic spectra remains an elusive and

TABLE 2. Matrix compounds and the types of lasers used in MALDI-TOF mass spectrometry of selected studies of whole cell bacteria

Lasers	Matrix	Reference	Comments
Nitrogen	SA	Easterling et al., 1998	
Nitrogen	SA	Nilsson, 1999	
Nitrogen	SA	Gantt et al., 1999	
Nitrogen	SA	Nilsson, 1999	
Nitrogen	SA	Ryzhov et al., 2000	Spores/CPD
Nitrogen	SA	Winkler, Uher, & Cepa, 1999	
Nitrogen	HPBA	Welham et al., 1998	
Nitrogen	DHBA	Fukuo et al., 1998	Purified Proteins
Nitrogen	DHBA	Bundy and Fenselau, 1999	
Nitrogen	DHBA/SA	Hathout et al., 1999	CPD
Nitrogen	FA	Madonna et al., 2000	
Nitrogen	FA	Saenz et al., 1999	
Nitrogen	FA	Jarman et al., 2000	
Nitrogen	FA/SA	Edwards-Jones et al., 2000	
Nitrogen	FA	Domin et al., 1999	
Nitrogen	HCCA	Erhard et al., 1999	
Nitrogen	HCCA	Easterling et al., 1998	Purified Proteins
Nitrogen	HCCA	Dai et al., 1999	
Nitrogen	HCCA	Wang et al., 1998	
Nd:YAG	HCCA	Chong et al., 2000	Purified Proteins
Nd:YAG	HCCA	Krishnamurthy and Ross, 1996	
Nd:YAG	HCCA	Holland et al., 1996	
Nd:YAG	HCCA	Holland et al., 1999	
Nd:YAG	HCCA	Holland et al., 2000	
Nd:YAG	HCCA	Arnold and Reilly, 1998	
Nd:YAG	HCCA	Arnold et al., 1999	
Nd:YAG	HCCA	Persson et al., 2000	

Studies involved MALDI-TOF/MS of whole cells, unless otherwise indicated. SA, sinapinic acid; HCCA, alpha-cyano-4-hydroxycinnamic acid; HPBA, 2,4-hydroxyphenyl benzoic acid; DHBA, 2,5-dihydroxy benzoic acid; FA, ferulic acid. CPD, corona plasma discharge.

perhaps controversial goal. This factor is evidenced by continuing and sometimes conflicting reports in the literature regarding optimum methodologies as various groups have refined their own procedures. Studies in addition to those described above have looked at other solvent or matrix effects (Domin, Welham, & Ashton, 1999), the use of internal controls to address the concentration dependence on bacterial spectra (Gantt et al., 1999), and the use of a two-layer sample preparation procedure for MALDI (Dai, Whittall, & Li, 1999). Concentrations that are either too high or too low can result in spectra with little or no indication of the presence of bacteria in the sample (Gantt et al., 1999). Remarkably, despite the differences of opinion regarding the best conditions, after about 4 years of MALDI studies using whole cells by a variety of investigators, all reports suggest that the method has demonstrated strong potential for bacterial characterization.

A few particularly novel approaches have been developed to increase the sensitivity or specificity involved in

the detection of bacteria by MALDI. The use of avidin-biotin technology to recover biotinylated proteins from bacteria has already been mentioned (Girault et al., 1996). Perhaps more noteworthy in the context of recovering whole cells is the Fenselau group's use of immobilized lectins incorporated into "affinity surfaces" to isolate several kinds of samples, including bacteria, for mass spectrometric analysis (Bundy & Fenselau, 1999). The recovery of bacterial species that have a carbohydrate-binding motif was demonstrated with a Concanavalin A probe. The probe was immobilized on a gold foil via a self-assembled monolayer. Then, urine, spiked with *E. coli* was placed into contact with the probe surface and was allowed to interact briefly. Just prior to MALDI/MS analysis, the capture surface was washed to remove salts and other unbound components. The lectin-derivatized surface allowed bacteria to be concentrated and readily characterized at levels that corresponded to about 5,000 cells applied to the capture surface. The target biomarkers in this study were not proteins, however, but rather

phosphatidylethanolamines, which yields masses below m/z 1000. Thus, this study demonstrates not only the utility of affinity processes for recovery of small numbers of bacteria from complex samples, but it also clearly shows that small molecules can play an important role as bacteria-specific markers in the characterization of bacteria by MALDI-TOF/MS.

Low level detection of spore-forming bacteria has also been reported (Hathout et al., 1999). Unique patterns of biomarkers from 1 to 10 kDa were reproducibly found to characterize and differentiate *Bacillus* species members, one from another, using less than 5,000 cells deposited on the target. In this same study, discrimination at the strain level was accomplished for *Bacillus cereus* spores. Lipophilic biomarkers, attributed to compounds present on the outside of the spore, were characteristic of *Bacillus* spores produced, using three different media and in spores stored for more than 30 years. Moreover, the “biomarker” character of the characteristic ions was supported by replicate characterization of spores at multiple sites and under different conditions. The nature of the *Bacillus* protein biomarkers were also characterized by MALDI analysis by using spores treated briefly with a CPD before analysis. The use of a CPD increased the specificity for MALDI characterization of bacterial spores in this study and also in another study that used spores from a larger number of *Bacillus* bacteria (Ryzhov, Hathout, & Fenselau, 2000). CPD prior to MALDI was compared to several other bacterial treatment regimes. Figure 9 shows spectra for *B. cereus* T spores suspended in 70% acetonitrile 0.1% FA without treatment, after 72 h of exposure to the bacterial suspension solution, after about 3 sec of CPD treatment, and after sonication at 20 kHz (maximum power) for a few minutes. As the top trace in Figure 9 shows, the untreated spores showed only a few peaks near m/z 4833. Spectra from the 72-h solvent treatment (second trace) showed small signals near m/z 6700. The CPD-treated sample showed strong signals at m/z 6712 and 6834 (third trace) very similar to the spectra from the sonicated spores (bottom trace). Spectra obtained directly from several *Bacilli*, including *B. anthracis*, *B. cereus*, and *B. thuringiensis*, also showed many spectral similarities without CPD, whereas *B. mycoides* was quite different. However, despite these similarities, the spectra were sufficiently unique to allow the differentiation of the different *Bacillus* species as well as *B. cereus* and *B. thuringiensis* strains with or without CPD. The number of detectable biomarker ions in the MALDI spectra of these spores generally increased upon treatment by CPD, thus simplifying differentiation. These authors noted, as exemplified in Figure 9, that CPD-treated bacteria often give spectra very similar to those obtained by sonication of spores prior to MALDI analysis. Spectra of sonicated and CPD-treated spores displayed a plethora of common

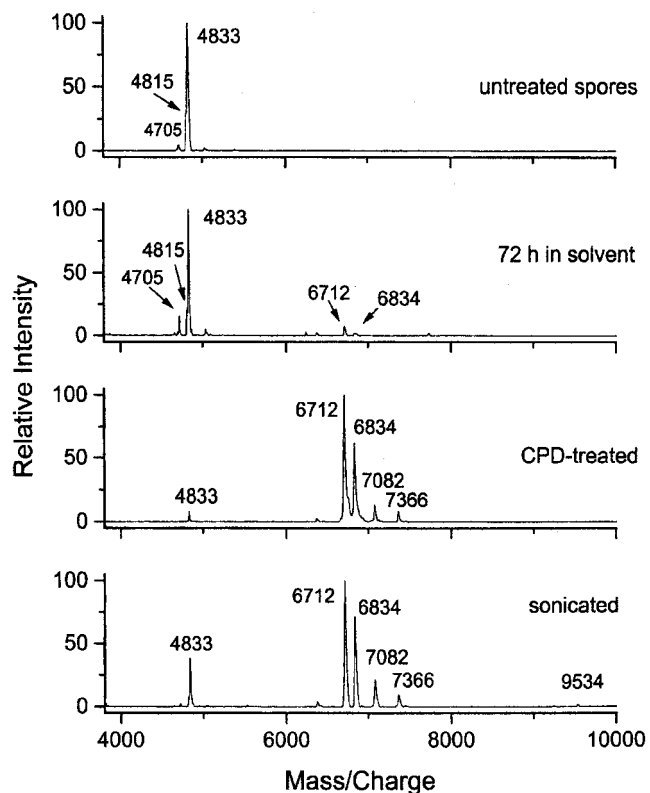


FIGURE 9. MALDI-TOF mass spectra from *B. cereus* spores suspended in solution (untreated), suspended for 72 h, treated with CPD, and sonicated. [Reproduced from Ryzhov, Hathout, & Fenselau (2000) with the permission of the American Society for Microbiology].

ions for the *B. cereus* group of bacteria. Fortunately, other biomarkers allowed differentiation of *B. cereus* group spores from each other, from *Bacillus subtilis*, and from *Bacillus globigii*.

VIII. INTERPRETATION OF DATA FROM WHOLE CELLS

As noted above, one of the key difficulties encountered in the use of MALDI-TOF/MS for the characterization of bacteria has been the interpretation of the data. The spectral dependence on experimental parameters includes a dependence on time-related factors such as bacterial growth and storage, and the fact that the number of proteins detected is affected by the separation of proteins into multiple fractions. Excepting the subset of conserved biomarker signals, these and other instrumental and biology-based spectral changes complicate the process of interpretation. Generally, pattern recognition methods require sufficient attention to experimental details so that reproducible spectra can be obtained from unknowns and

reference cultures acquired under the specified conditions. The alternative approach suggested above involved the more selective use of a smaller set of “conserved” biomarker proteins, based on the assumption that even a few consistently observable marker proteins could be used to provide adequate specificity. Exactly how conserved and unique these bacteria-specific ions are, and how they can be identified and best utilized, has been addressed in several experiments. Jarman et al. developed a method to construct and extract reproducible MALDI spectral from whole cells. Their method is based on statistics, automated data extraction, and a relatively high level of spectral reproducibility (Jarman et al., 1999, Jarman et al., 2000). The method can be used to extract a fingerprint (specific biomarkers) characteristic of a targeted bacterium from a complex spectrum that contains multiple analytes or other background signals. They illustrated their approach in a series of studies using *E. coli* and *B. atrophaeus* (Jarman et al., 2000) and then using *B. atrophaeus*, *B. cereus*, *E. coli*, *Pantoea agglomerans*, and *Pseudomonas putida* (Jarman et al., 2000). This method was demonstrated using blind-coded samples with a limited number of strains. It clearly demonstrated the feasibility of automated data interpretation for some applications.

An alternative method that exploits the information contained in prokaryotic genome and protein sequence databases has been proposed (Pineda et al., 2000). This method is based on determining the masses of a set of protein ions by MALDI-TOF mass spectrometry, using intact cells, treated cells, or from fractions. Subsequently, the correlation of each ion in the set to a protein, along with the organism specific source of the protein, is accomplished by searching an internet-accessible protein database. Convoluting the lists for all ions and ranking the organisms that correspond to matched ions allows the identification of the organism. This approach was demonstrated, using two bacteria, *B. subtilis* and *E. coli*, two organisms that have completely sequenced genomes. The method was also tested with mixtures of microorganisms, using spectra from an organism at different growth stages, and from spectra that originated in other laboratories. This method has several advantages over other pattern-recognition methods for microorganism identification. Efficacy is based upon the presumption that, although spectra might change in concert with changes in the experimental conditions, as long as any bacteria-specific set of proteins can be associated uniquely with the correct set of predicted proteins, identification should be possible. This approach has some disadvantages as well. Only a limited number of bacteria exist for which the genome is known. Higher accuracy in the mass assignment step is also needed to minimize the possible number of protein assignments for each mass. Finally, and perhaps

most importantly, a complex variety of the biological processes can cause the protein masses to differ from those masses predicted from the genome.

IX. IDENTIFICATION OF PROTEINS AND METABOLITES IN CELLS

A few studies have reported on the identification of the bacterial proteins that give rise to the signals in the MALDI-TOF mass spectra from whole cells. The identification of specific biomarkers might enhance their value for taxonomic identification, especially if they are from a conserved set of biomarkers. Proteins that are associated with specific properties can also be used as markers for these properties and to detect the presence of specific genes. Holland et al. (1999) have reported on the identification of several proteins, observed from cells and in filtered cellular suspensions, which were isolated by HPLC and identified on the basis of their mass spectra and their partial amino acid sequence, determined using the Edman method. Two of these proteins included the acid-resistance proteins HdeA and HdeB (ions near m/z 9735 and 9060) from whole cells and cell extracts of *E. coli* 1090 and *Shigella flexneri* PHS-1059. Interestingly, although these proteins were detected, they were not identified in another study that used a peptide mapping approach (Dai et al., 1999). This difference might have occurred because the specific proteins detected by Holland et al. resulted from the biological processing of the parent protein within the cell. In this case, in-cell proteolytic cleavage by the peptidase Lep gave smaller proteins than expected based only on the genome. This experiment demonstrated the detection of a pair of virulence-related biomarker proteins that were identical in two different organisms. Perhaps more important, it also illustrated the need to take into account cellular processing steps when assigning identities to proteins. This factor must also be taken into account in schemes that involve comparisons of lists of MALDI masses with masses predicted from the genome. In this same study, a cold-shock protein, CspA, was associated with an ion near m/z 7643 from *Pseudomonas aeruginosa* and a cold-acclimation protein, CapB, was identified as the source of the ion near m/z 7684 in *P. putida*. This last protein was homologous with a known CapB from *P. fragi*.

These same types of proteins have been monitored with MALDI in other studies, but not directly from cells. The identification of a cold shock protein from an *E. coli* isolate via peptide mapping (Dai et al., 1999) was mentioned in Section V. Another study specifically targeted the detection of cold shock proteins. HPLC separation was used to produce fractions for analysis by MALDI-TOF/MS analysis, and to detect the increased expression of

cold shock proteins in bacteria collected from Siberian permafrost (Chong et al., 2000). Distinct protein profiles were observed from cultures as a function of temperature. Peptide maps of the proteins were generated by tryptic digestion. Although these experiments involved the detection of known or homologous proteins, this same approach could also be applied to the detection of unique proteins. In another study, the acid response of the organism and the correlated protein synthesis were studied in *Listeria monocytogenes* (Phan-Thanh, Mahouin, & Alige, 2000). The bacterial strains that survived acid treatment produced more stress proteins under severe acidic conditions than normal bacteria. In this last set of experiments, two-dimensional electrophoresis was used prior to MALDI.

Secondary metabolites have been characterized coincident with bacterial identification of whole cells using MALDI. Toxic cyanobacterial blooms are a threat because of secondary metabolite production, and the detection of the toxins may be as important as finding of the organisms themselves. MALDI-TOF mass spectrometry was used to identify intact cyanobacteria and to detect their secondary toxic metabolites. These included the microcystins, micropeptin, and anabaenopeptin (Erhard, von Dohren, & Jungblut, 1997, 1999). Figure 10 shows the MALDI-

TOF mass spectrum of *Microcystis aeruginosa* collected from a water bloom. The protonated molecule ions of several known peptides were detected, including microcystins (m/z 981, 995, and 1038), anabaenopeptin B and F (m/z 837 and 851), and a new microcystin-LR derivative (m/z 1013). A new cyclic anabaenopeptin from *Planktothrix agardhii* HUB 011 was also identified using CID and PSD. These authors reported that strains of various origins were typed according to the profile of their cyclic peptides, and that toxic and nontoxic algal blooms were differentiated within minutes. It should be noted that, although most other MALDI-TOF/MS studies of intact cells have focused on peptides with masses above 2 kDa, Erhard, von Dohren, & Jungblut (1999) demonstrated that smaller peptides can also be used as characteristic biomarkers, especially when they are themselves of interest as bioactive bacterial toxins.

In another interesting study, pigments and proteins from chlorosomes, the light-harvesting organelles from the photosynthetic green sulfur bacterium *Chlorobium tepidum* were characterized directly from organelles (Persson et al., 2000). By applying a small volume of a concentrated suspension of isolated chlorosome organelles directly onto the target, bacteriochlorophyll a and all the major homologs of bacteriochlorophyll c were

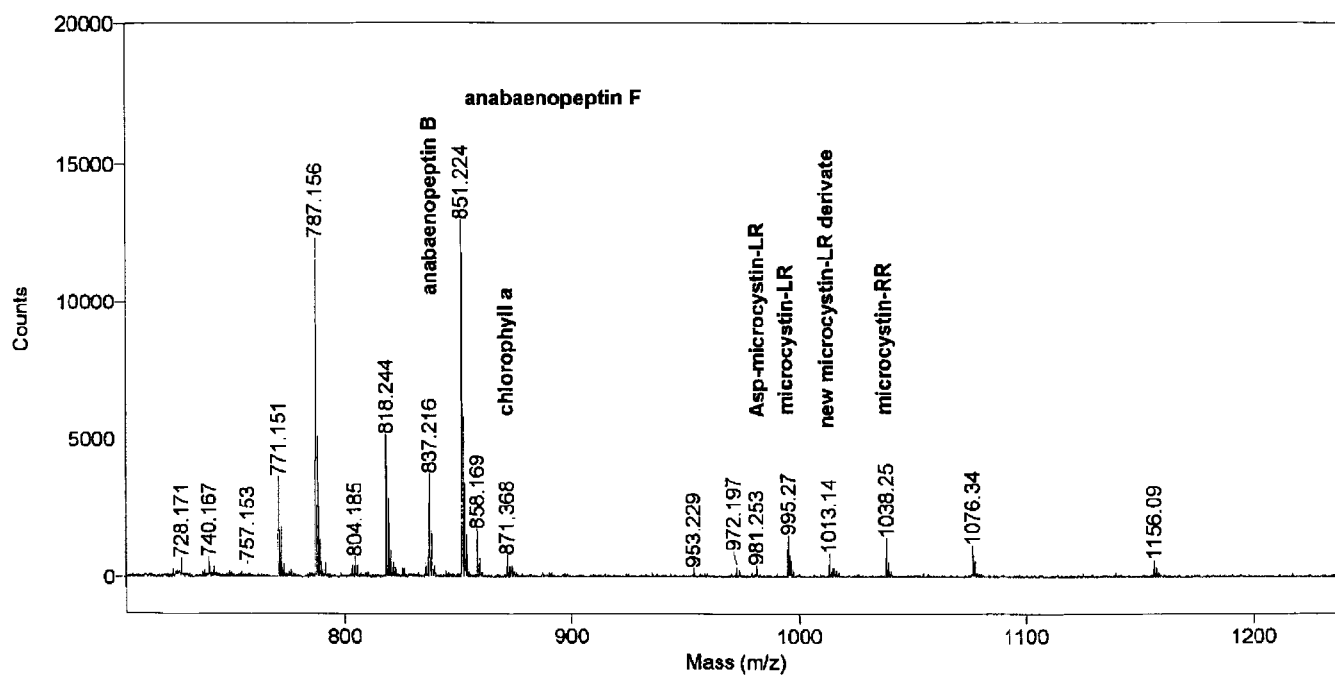


FIGURE 10. Lower mass range MALDI-TOF mass spectrum (m/z 700–1250 Da) of *M. aeruginosa* cells from a water bloom. (A low-mass cut-off and the reflectron mode of operation were used to minimize the usual large contribution from the matrix in the lower mass region displayed in the spectrum). [Reproduced from Erhard, von Dohren, & Jungblut (1999) with the permission of John Wiley].

detected. The authors noted that the peak heights of the different bacteriochlorophyll *c* homologs in the MALDI spectra were proportional to peak areas obtained by HPLC analysis. Similar results were reported when whole cells of *Chl. tepidum* were applied to the target. These results suggest that MALDI-TOF/MS can rapidly provide semiquantitative analysis as well as a fingerprint for the small amount bacteriochlorophyll homologs present in cells from green bacteria. The MALDI spectra also contained peaks attributed to chlorosome proteins. The molecular masses of the chlorosome proteins CsmA and CsmE previously determined by conventional biological methods were confirmed, as was the presence of truncated versions of the chlorosome proteins CsmA and CsmB.

X. ISOLATE-RELATED STRAIN DIFFERENCES IN HUMAN PATHOGENIC BACTERIA

Species of the genus *Haemophilus* are important etiological agents that cause pneumonia, meningitis, conjunctivitis, epiglottitis, and chancroid. However, the identification and speciation of *Haemophilus* in clinical settings has been time-consuming and expensive. The characteristic spectra obtained from whole cells have suggested that MALDI-TOF/MS might emerge as a powerful tool for diagnostic bacteriology. A paper by Haag et al. (1998) reported the use of MALDI-TOF/MS as a technique for the rapid identification and speciation of pathogenic *Haemophilus* strains. They used MALDI to identify a pathogen, *H. ducreyi*, distinct from other genus and species, and also reported strain differences from different isolates of this organism. Their mass spectral 'fingerprints' permitted the rapid speciation of not only the pathogenic forms of *Haemophilus*, but also the non-pathogenic strains and other members of the normal human flora.

H. pylori represents another major human health problem. This organism plays an important role in human gastrointestinal health, and is believed to colonize approximately one-half of the world's population. Strain differentiation is important for this organism because some strains of *H. pylori* possess virulence proteins for tissue colonization, host evasion, and tissue damage, whereas others do not. The organism displays genomic instabilities that include gene rearrangements and gene exchange, leading to frequent strain-related differences. A set of presumed strain-specific biomarkers were detected based on the MALDI-TOF/MS analysis of lysates and extracts from six different strains of *H. pylori* (Nilsson, 1999). The author proposed that *H. pylori* fingerprinting by MALDI might allow typing and studies of genetic drift at the phenotypic level from human isolates.

An increasingly important set of human-health related differences among bacteria results from their emerging resistance to antibiotics, bactericides, and sterilization procedures. Progress has already been reported on the differentiation of antibiotic resistant and non-resistant strains of one important human pathogen, *Staphylococcus aureus* (Edwards-Jones et al., 2000). The rapid and accurate discrimination between methicillin-sensitive and methicillin-resistant strains of this organism (and others) could lead to major improvements in the treatment strategy for infected patients. This specific strain-specific differentiation by MALDI-TOF mass spectrometry was demonstrated from intact cells from 20 *Staphylococcal* isolates. The spectra showed characteristic peaks from 500 to 10,000 Da with species-specific and strain-specific markers as well as characteristic markers for the methicillin susceptibility status of the strain being detected.

XI. CONCLUSION AND FUTURE PROSPECTS

It is now possible to evaluate the reproducibility and chemotaxonomic utility of the MALDI-TOF/MS method using spectra generated over a period of years. Current data support claims regarding the existence of sets of bacteria-specific conserved biomarker ions. To cite a single example, Lynn et al. (1999) reported MALDI-TOF/MS spectra for several bacteria that had previously been analyzed. Their spectra reproduced a number of specific marker ions claimed as species or strain-specific for these organisms in the work of others. Indeed, the field is sufficiently mature so that reviews are beginning to appear in the microbiology literature (van Baar, 2000). Some additional work remains to be done to address difficulties with data interpretation and manipulation, the creation of reference libraries, and to facilitate spectral comparisons. This is all complicated by not-unusual shifts in mass assignments that result from overlapping peaks and the large number of data points present in individual spectra. Work also needs to be done to facilitate database searches using simple approaches to account for differences between observed and predicted masses that result from biological processing within the cells. The biological origin and general nature of the ions observed in MALDI spectra from bacteria are now fairly well-established. Ryzhov & Fenselau (2000) have shown, using *E. coli*, that the MALDI-produced ions are predominantly associated with strongly basic proteins, moderately hydrophilic in nature, likely coming from the cytoplasm and organelles such as the ribosome.

One of the most profound limitations on the use of MALDI for the characterization of bacteria is the fact that the technique's greatest advantage, namely the speed of analysis, seems to be mitigated by the usual practice of

amplifying cells in a culture medium prior to analysis. Irrespective of the fact that competing molecular biology techniques also rely heavily on preliminary cell culture steps prior to DNA amplification, the development of approaches that do not depend upon preliminary culture steps would greatly increase interest in this approach. One example of such an approach, involving the use of affinity-capture surfaces to selectively capture the bacteria present in a complex sample (Bundy & Fenselau, 1999), has already been described in Section VII. Taking another approach, Holland et al. (2000) demonstrated the analysis of bacteria mechanically collected directly from contaminated media without pre-MALDI culture steps. Spectra of bacterial proteins were obtained from water, lettuce, and cloth samples contaminated with *S. flexneri*, *E. coli*, and *A. hydrophila*. For *S. flexneri* and *E. coli*, the two virulence-marker ions for acid-resistance (HdeA and HdeB noted in Section IX above) were readily detected. As in another study (Madonna et al., 2000), a sample contaminated with two bacteria showed protein biomarkers from both bacteria. Although the experiments described above involved relatively large numbers of bacteria, the taxonomic characterization of bacteria has been demonstrated by Fenselau's group using two different methods, using as few as 5,000 organisms (Bundy and Fenselau, 1999; Hathout et al., 1999). Traditional MALDI experiments with even smaller numbers of cells should be possible with the emergence of new technologies and affinity-capture strategies. One approach worth specific mention is based on the so-called "chip" technology. This concept has led to gene-chip and protein-chip methods that allow the characterization of complex samples for very large numbers of genes or proteins, using very small or very dirty samples. Although much of the thrust of these technologies has been directed towards human therapeutics and diagnostics, their application to high-throughput characterization of bacteria may lead to direct characterization of bacteria or bacterial proteins from chips with MALDI. One application of this general sort has already been reported for *E. coli*. Nelson et al. (1999) reported on their use of biomolecular interaction and MS to selectively isolate, detect, and characterize epitope-tagged peptides present in bacterial cell lysates. Epitope-tagged tryptic peptides were captured via affinity interactions with either chelated Ni²⁺ or monoclonal antibodies and detected using surface plasmon resonance biomolecular interaction analysis (SPR-BIA). After SPR-BIA, the tagged peptides were either eluted from biosensor chips or they were analyzed directly from the biosensor chip, using MALDI-TOF/MS. When protein database searches were performed using the masses of the tagged tryptic peptides, they reported that the protein into which the epitope tag was inserted was detected. Moreover, the detection limits for SPR-BIA and

MALDI-TOF/MS both ranged from the low-femtomole to the subfemtomole level. It seems likely that similar strategies will be incorporated into the production of bacteria-specific chips for direct MALDI-TOF/MS analysis in the not too distant future.

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