Use of Double-Depleted ^{13}C and ^{15}N Culture Media for Analysis of Whole Cell Bacteria by MALDI Time-of-Flight and Fourier Transform Mass Spectrometry

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In the present paper, results demonstrating the significant advantages of matrix-assisted laser desorption/ionization (MALDI) analysis of whole cell samples of bacteria grown on double isotopically-depleted (^{13}C and ^{15}N) media are presented. It is shown that several advantages accrue for MALDI with a 9.4 T Fourier transform mass spectrometer (FTMS). Of particular note, for analysis of whole cells, sample preparation is simple and chemical interference is reduced. Moreover, ion coalescence problems are minimized, and data-base identification of proteins facilitated. Furthermore, high resolution mass spectra obtained from such whole cells show significant improvement in apparent mass resolving power and mass measurement accuracy, whether time-of-flight or FTMS MALDI is used. As a consequence, it becomes possible to detect subtle details in the chemistry of the organism, such as the presence of both post-translationally modified and unmodified versions of the same proteins. This approach is also adaptable to direct assay of over-expressed proteins from *Escherichia coli* cultures and should facilitate studies aimed at the detection of medically important cellular biomarker proteins. (J Am Soc Mass Spectrom 2003, 14, 1306–1314) © 2003 American Society for Mass Spectrometry

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Accurate determination of molecular masses of cell constituents for complex samples such as whole cell bacteria is a challenging analytical problem. When high resolution mass spectra of proteins or other materials with molecular masses above a few thousand Daltons are involved, the seemingly simple determination of even the monoisotopic molecular mass can be as much as 1 Da or more in error [1]. Several years ago Marshall and co-workers demonstrated the considerable advantages of using electrospray Fourier transform ion cyclotron resonance (ESI-FTICR) to analyze a protein from an *Escherichia coli* grown in a medium in which ^{13}C and ^{15}N were depleted [2]. Zubarev and Demirev further discussed the implications of this approach for molecular mass measurements [3]. Later ESI-FTICR studies of lysates of *E. coli* grown in this way also showed the improved mass measurement accuracy possible by utilizing such a strategy [4]. Further such studies demonstrate use of lysates of cultures grown on rare isotope-depleted me-

dia as effective internal calibrants in protein expression investigations [5].

This paper is the first report of results demonstrating the significant advantages of MALDI analysis of whole cell samples of bacteria grown on ^{13}C and ^{15}N depleted media. In addition to the substantial improvements previously demonstrated with high performance ESI-FTICR, several advantages accrue for MALDI with a 9.4 T Fourier transform mass spectrometer (FTMS). For analysis of whole cells, sample preparation is simple, the previously-mentioned chemical interference is eliminated, ion coalescence problems [6, 7] are minimized, and data-base identification of proteins facilitated. Furthermore, high resolution mass spectra obtained from such whole cells show significant improvement in mass resolving power and mass measurement accuracy, whether time-of-flight or FTMS MALDI is used. As a consequence, it becomes possible to detect subtle details in the chemistry of the organism, such as the presence of both post-translationally modified and unmodified versions of the same proteins. Furthermore, as shown below, *vide infra*, this approach is also adaptable to direct assay of over-expressed proteins from *E. coli* cultures.

Analysis of whole bacterial cells by matrix-assisted laser desorption/ionization (MALDI) time-of-flight

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(TOF) mass spectrometry (MS) is of interest [8–15]. Several recent review articles have summarized the current status [16–19]. One aspect of particular interest is rapid identification of bacterial cells [8–11, 15, 20, 21]. However, despite the promise of MALDI for rapid bacterial analysis with minimal sample preparation there are still several major concerns, such as the reproducibility of spectra, data analysis, and identification of potential “biomarkers”. Biomarkers, unique proteins specific to a particular bacterium, are especially useful for taxonomic classification of bacteria because their presence can be used for identification even when whole-cell bacterial spectra show significant variability in ion abundances because of subtle changes in cellular biology.

Spectral reproducibility has been addressed by numerous groups [22–26]. Arnold and co-workers [27] using MALDI TOF MS, showed that different spectra can be obtained from sampling an organism at different times during the culture growth cycle. However, they also demonstrated that several proteins were conserved over the life cycle of a bacterium. Their conclusions argued for consistent growth conditions in order to maintain reproducibility. Long and coworkers [22] took this idea one step further by exploring the possibility of reproducibility over separate laboratories using the same experimental protocols and materials. They established that near identical spectra can be produced under carefully controlled conditions. It may be a more realistic expectation that only certain prominent biomarkers are conserved from laboratory to laboratory. As shown in the present study, such a goal is much more likely to be achieved when the tactical use of appropriate isotopically depleted growth media is part of the analytical scheme. If certain protein biomarkers are used for chemotaxonomy, it is important that they be identified accurately and reproducibly. There are already literature examples of the ions that appear to result from a single *E. coli* protein being attributed in various laboratories to different proteins [22, 28–30]. This is a not-unexpected result of searching protein databases using nominal mass values.

Thus, spectral data interpretation and analysis is a pertinent concern in the application of MALDI to bacterial identification [31–35]. Because, in contrast to capillary electrophoresis approaches [4], whole cell MALDI techniques include no separation prior to analysis, complex protein mixtures are expected. To date, the observed spectral patterns are less complex because only the most abundant proteins, representing just a fraction of the cellular proteins (typically ribosomal) are observed. Nevertheless, because the pool of proteins being sampled is very large, data analysis and interpretation is still challenging [30, 34]. The availability of genome-based protein databases for bacteria does not simplify matters a great deal. For example, although for *E. coli* post-translational methionine loss is normally expected, results from Reilly's group demonstrate that C-terminal methionine can be retained or lost, perhaps

depending on the next amino acid in the sequence [29]. Fenselau and coworkers [36] have reported that there are post-translational modifications by prokaryotic organisms that considerably complicate the analysis. Additionally, Holland, et. al. [37], have shown that the proteins in cells can result from in-cell proteolytic processing to give parts of proteins not readily detected from database searches using the mass values from MALDI spectra. These protein modifications can lead to any number of differences between the predicted and observed proteins that can prevent the correct identification of biomarkers assigned to mass spectral peaks. Approximately 15% of the dry weight of a bacterium is protein, and 20% of the proteins within the cell are ribosomal [38]. Thus, observations that most of the bacterial proteins detected in whole bacteria are from the ribosomal fraction are not unexpected. However, assignment of specific post-translational modifications to these proteins in whole cell spectra based on nominal mass values (from TOF) is not warranted. Using a ribosomal fraction (not whole cells) Arnold and Reilly [29] observed that several ribosomes seem to be present without the expected post-translational modifications, most notably the loss of the C-terminal methionine. Recently, we have confirmed the identity of several ribosomes and their modified forms by accurate mass, high resolution MALDI FTMS analysis of whole cell *Escherichia coli* [39]. Because the FTMS measurements were done with high accuracy, reliable assignments of the detected ions to ribosomes are possible even without separation from the other cellular proteins. However, in our prior work we were not able to detect more than seven ribosomal proteins from MALDI of *E. coli* whole cells. Moreover, the identities of other, non-ribosomal proteins could not be confirmed.

As mentioned at the outset, this paper demonstrates the use of doubly-depleted (^{13}C and ^{15}N) culture media to increase the numbers and types of proteins that can be detected directly from whole cells. The increased number of proteins detected can be attributed to a reduction in chemical noise, greatly improved mass accuracy, and an increase in the sensitivity of ion detection for MALDI FTMS experiments. Similar improvements also are seen in MALDI TOF studies on the same bacteria. The improved sensitivity results from most of the mass spectral signals being concentrated into a single isotopic peak for each protein, allowing spectra to be obtained with fewer ions in the cell, ultimately improving FTMS performance. Collection of predominantly monoisotopic spectra reduces the magnitude of space charge effects [40, 41] and peak coalescence [6, 7] as the mass difference between ions confined within the cell becomes larger. Moreover, by simplifying the isotope profiles and concentrating ion population into monoisotopic peaks for each molecular species, it becomes possible to more accurately ascertain the mass of those species.

Experimental

Instrumentation

TOF measurements. All experiments were performed using a Bruker Reflex III MALDI TOF mass spectrometry (Bruker Daltonics, Billerica, MA) and an external ionization Fourier transform mass spectrometer (Ion Spec Corp., Lake Forest, CA) equipped with 9.4 Tesla superconducting magnet. Both systems utilized nitrogen lasers (Laser Science, Mountain View, CA) operating at $\lambda = 337$ nm. MALDI TOF experiments were performed in reflectron mode using a delayed extraction pulse in the source region with a 20.0 kV acceleration voltage and a 23.0 kV reflecting potential. The detector is a dual-multichannel plate detector with a detection voltage of 1.55 kV. Base pressure during analysis is $1.0\text{--}2.0 \times 10^{-8}$ torr in a vacuum chamber that is differentially pumped by two turbomolecular pumps.

FTMS measurements. FTMS experiments were conducted in positive ion mode with ions being generated externally, and then focused using a quadrupole ion guide to gate the ions into the 15.24 cm long by 5.08 cm diameter analyzer cell. Initially, ions are removed from the cell by applying a 9 V (b-p) quench potential for 5 ms to all analyzer plates. Then, a 20 V (b-p) potential is applied to the outer cell plates to decelerate ions injected from the quadrupole ion guide. A series of seven laser shots spaced 100 ms apart are used to generate ions from the MALDI probe surface. The quadrupole ion guide has a 719 kHz radio frequency, 495 V (b-p) potential applied to the coil for 650 ms beginning 30 ms before the initial laser pulse. Laser-desorbed ions are cooled by a pulse of nitrogen gas from a 40 mTorr reservoir that is introduced for 700 ms beginning 50 ms after the quench event. The ions are then allowed to relax in the cell for 1.3 s, and then the potential of the analyzer cell is ramped down from 20 to 0.5 V over a 1000 ms interval. For ion excitation, a chirp excitation is applied for 2.5 ms at 150 V (b-p) over the frequencies corresponding to the range m/z 400–14,000. Detection is over a broadband mass range of m/z 2000–13,000 at an ADC rate of 200 kHz, acquiring either 1 M or 2 M of data points for total transient durations of 5.243 s or 10.486 s. To enhance signal to noise ratio, except as noted, the first 512 K data points of the acquired data set were processed to yield the frequency domain spectra. Average base pressure during detection as read by the ion gauge is $1.0\text{--}3.0 \times 10^{-10}$ torr.

Calibration

Internal FTMS calibration was performed by using the following procedure to accurately assign masses to broadband spectra. Monoisotopic ion species from five of the previously-identified ribosomal proteins [39] were used as calibrants. The broadband spectra were divided into two mass ranges (m/z 2000–7000 and m/z

7000–13000) and the calibrant peaks falling within the ranges used to separately calibrate those subspectral regions.

Sample Preparation

Escherichia coli JM109 (obtained from University of Arkansas Microbiology Department) cultures were grown in mono-express natural abundance media (Cambridge Isotope Laboratories, Inc., Andover, MA) and mono-express cell growth media (Cambridge Isotope Laboratories, Inc.), which is doubly-depleted of ^{13}C and ^{15}N isotopes ($>99.95\%$ ^{12}C and $>99.97\%$ ^{14}N). Stock cultures of *Escherichia coli* JM109 were used to inoculate 10 mL portions of mono-express media that were then incubated at 37 °C for twenty h.

Bacteria grown in mono-express culture media were combined into 50 mL centrifuge tubes and spun down at approximately 500k RCF (relative centrifugation force) for 20 min. The supernatant was poured off and 10 mL TRIZMA buffer solution (Sigma, St. Louis, MO) at 35 °C was added to the sediment. The sediment was then vortexed to resuspend the bacteria. The cells were again concentrated by centrifugation at approximately 500k RCF for 20 min. The supernatant was poured off and 1.0 mL of 35 °C TRIZMA buffer solution was added and the cells resuspended. The concentrated cells were then transferred to 0.5 mL vials. The vials were vortexed, then centrifuged at 25k RCF for 10 min to concentrate the cells. The supernatant was removed and 0.5 mL of a solution of 50/50 methanol and TRIZMA buffer was added. A small portion of this cell suspension was deposited within 10 min of workup onto a MALDI surface for analysis.

MALDI

All spectra were obtained using a saturated solution of 2,5-dihydroxybenzoic acid (DHB), dissolved in 100% methanol. The probe surface in both instruments is stainless steel, and was cleaned after each spectral measurement. A “sandwich” preparation procedure is used. Sample was prepared by first applying 1 μL of matrix solution to the probe surface and allowing it to dry. Next, 1 μL of the bacteria suspension was added and allowed to dry. Then, 1 μL of a 10% trifluoroacetic acid solution in deionized water was added and allowed to dry. Finally, another 1 μL of matrix solution was applied to the surface and allowed to dry.

Results and Discussion

TOF

As expected, cells from cultures grown on natural abundance media provided spectra similar to those that have been previously reported [13, 28–31, 37]. In the present study, within the fingerprint region of 4–12 kDa TOF spectra containing thirteen of those ribosomal

Figure 1. Partial reflectron time-of-flight *E. coli* JM-109 MALDI spectra over the range m/z 6200–6270. (a) Cells grown on natural abundance media; (b) cells grown on isotopically-depleted media.

proteins are observed. The main problem lies with reliable identification of the individual proteins. Optimum reflectron-TOF resolving power for whole cell samples desorbed in this mass region is about 5000 for masses below 5000 Da. Above that mass, routine resolving power of 1500 is obtained. With these resolving powers, peak overlap cannot be avoided. Therefore, mass measurement accuracy is degraded.

The cells from cultures grown on isotopically depleted media provide spectra that show the same proteins as the natural abundance media, except that ions with masses containing ^{13}C and ^{15}N are very much decreased in abundance. For example, one ion peak with m/z of 6254.7 (over 100 ppm error) was identified as the singly-methylated L33 50S ribosomal protein, with the Swiss-Prot [42] accession number P02436. Its chemical formula is $\text{C}_{285}\text{H}_{478}\text{O}_{76}\text{N}_{81}$ with an average mass of 6255.35 Da and a monoisotopic mass of 6251.6027 Da. With cells from isotopically depleted media, the observed mass is 6251.7 Da (15 ppm error). Figure 1 shows this increase in apparent resolving power and sensitivity associated with use of isotopically depleted media in the mass region 6220–6270 Da. Figure 1a is a spectrum from *E. coli* grown in natural abundance media and Figure 1b is the spectrum from *E. coli* grown in isotopically depleted media. Additionally, the latter spectrum shows the presence of the unmethylated version of the protein (theoretical m/z of 6237.5872) that is not observed in the spectrum from cells grown in natural abundance media. This is an excellent example of the value of removing chemical noise due to isotopic multiplicity.

FTMS

Figure 2 shows a spectrum covering the region from m/z 2000–10,000 from *E. coli* grown in natural abundance media. Ten transients, each obtained as described in the Experimental section, were summed to provide the raw data for this spectrum. Figure 3 is the corresponding spectrum from *E. coli* grown in isotopically depleted media. Due to the superior signal to noise ratio, only a single transient was required to obtain this spectrum. In a previous study, seven ribosomal proteins were identified by MALDI-FTMS [39]. Now, thirteen additional proteins are identified with the use of the isotopically depleted media (see Table 1). The significantly simplified data resulting from using depleted media for bacteria culture facilitates studies of bacteria proteomics. As one example, consider the methylated L33 50S ribosomal protein mentioned earlier. Figure 4a and b are expanded plots over the range m/z 6200–6270 from the spectra of Figures 2 and 3. Figure 4a, which is the FTMS spectrum from cells grown on natural abundance medium, allows identification of the methylated parent protein as well as what appear to be a series of water loss products. The isotopic multiplicity obscures most other details. However, the FTMS spectrum in Figure 4b for the bacteria grown on rare isotope depleted media, reveals additional information. First, a peak at 6237.584 corresponds to the unmodified ribosomal protein whose monoisotopic mass is 6237.5872 Da, meaning that it is possible to see both the modified and unmodified protein directly from whole cells in a single spectrum. Furthermore, the associated masses that were

Figure 2. Broadband MALDI-FTMS spectrum of *E. coli* JM-109 cells grown on natural abundance media.

18 Daltons to the left of the parent ions were previously thought to be water losses. With the simplified isotopic multiplets, it is now clear that ammonia and water losses are present for both the unmethylated and methylated parent ions, further confirming identification of the original proteins.

Another region of interest is that between m/z 7268–7275. According to the Swiss-Prot database *E. coli* has

eight proteins within this region. Most notably, three of these proteins are cold shock Protein A, cold shock Protein C, and 50S L29 ribosomal protein, all with average masses that differ by less than 2.3 Da. Several researchers have identified masses found in spectra over this region as one of these three proteins, causing some controversy [28–30]. Figure 5 is a partial spectrum over the region 7200–7310 Da from *E. coli* grown under

Figure 3. Broadband MALDI-FTMS spectrum of *E. coli* JM-109 cells grown on isotopically-depleted media.

Table 1. List of theoretical and experimental masses of ribosomal and DNA-binding proteins from FT-MS analysis and their associated errors^{a,b}

Protein ID	Accurate mass (Da)	Observed mass (Da)	Δ (Da)	Error (ppm)	Comment
50S <i>E. coli</i> ribosome L36 P21194	4362.458	4362.447	0.011	2.5	Methionine retained
30S <i>E. coli</i> ribosome S22 P28690	5093.773	5093.758	0.015	2.9	Methionine retained
50S <i>E. coli</i> ribosome L34 P02437	5378.084	5377.977	0.107	19.9	Methionine retained
50S <i>E. coli</i> ribosome L33 P02436	6237.587	6237.570	0.017	2.7	–
50S <i>E. coli</i> ribosome L33 P02436	6251.603	Calibrant	–	–	Methylated
50S <i>E. coli</i> ribosome L32 P02435	6312.399	Calibrant	–	–	–
50S <i>E. coli</i> ribosome L30 P02430	6407.613	Calibrant	–	–	–
50S <i>E. coli</i> ribosome L35 P07085	7154.241	7154.235	0.006	0.8	–
50S <i>E. coli</i> ribosome L29 P02429	7269.991	Calibrant	–	–	Methionine retained
50S <i>E. coli</i> ribosome L31 P02432	7866.924	7866.988	0.064	8.1	Methionine retained
30S <i>E. coli</i> ribosome S21 P02379	8364.694	8364.750	0.056	6.7	–
50S <i>E. coli</i> ribosome L28 P02428	8870.841	8870.908	0.067	7.6	–
50S <i>E. coli</i> ribosome L27 P02427	8988.849	8988.880	0.031	3.4	–
30S <i>E. coli</i> ribosome S16 P02372	9185.958	Calibrant	–	–	–
DNA-binding protein HU-beta P02341	9221.002	9220.989	0.013	1.4	Methionine retained
DNA-binding protein HU-alpha P02342	9530.197	9530.152	0.045	4.7	Methionine retained
30S <i>E. coli</i> ribosome S20 or 50S L26 P02378	9548.288	9548.225	0.063	6.6	–
30S <i>E. coli</i> ribosome S15 P02371	10132.474	10132.420	0.054	5.3	–
30S <i>E. coli</i> ribosome S19 P02375	10293.682	10293.620	0.062	6.0	–
50S <i>E. coli</i> ribosome L25 P02426	10687.728	10687.650	0.078	7.3	Methionine retained
Average	–	–	0.046	5.7	–

^aMasses result from the average of ten replicate spectra. Average standard deviation of reported masses is 0.042 Da.^b**Bold print** designates the previously identified proteins used for internal spectrum calibration [39].

conditions which would favor expression of cold shock proteins. Although the P02429 ribosomal protein is clearly the most abundant, there is also evidence for presence of cold shock Protein C (although the parent ion is missing, decomposition product ions are present).

Figure 6 is a partial spectrum over the mass region 9140–9240 Da derived from a frequency domain spectrum obtained by transforming the entire 2 M time domain data acquired, rather than 512 K as was the case for the other spectra. The significance of this region is that the 30S S16 ribosomal protein is almost exactly 35 Da lower in mass than the DNA-binding protein HU- β , a difference which corresponds to a water plus an

ammonia loss from the DNA-binding protein. With samples grown in natural abundance media, it is difficult to determine if ribosomal protein is present. However when bacteria are grown with isotopically depleted media it is apparent that the ribosomal protein is present as well as the DNA-binding protein. Now, water and ammonia losses from the second DNA-binding protein are easily resolved from those of 30S S16 ribosomal protein. Similar observations are seen in the 9530–9555 Da region (not shown) except that now, the water loss peaks from the ribosomal protein 30S S20 overlap with the parent ion peak of DNA-binding protein HU-alpha.

Figure 4. Partial FTMS *E. coli* JM-109 MALDI spectra over the range m/z 6200–6270. (a) Cells grown on natural abundance media; (b) cells grown on isotopically-depleted media.

Conclusions

The use of isotopically-depleted growth media as applied to analysis of bacterial whole cells results in TOF and FTMS spectra showing apparent increases in resolving power as a result of reduction of chemical noise. There are also significant gains in sensitivity over spectra from bacteria grown on natural abundance

growth media. For both FTMS and TOF analysis, enhanced sensitivity permits much improved mass measurement accuracy. These results establish that use of isotopically-depleted growth media with MALDI is clearly the method of choice for whole cell chemotaxonomy. The average mass measurement error of the thirteen newly identified proteins is 5.7 ppm. With

Figure 5. Partial FTMS *E. coli* JM-109 MALDI spectra over the range m/z 7200–7310. Cells were cultured under cold-shock expression conditions [43]. Cells also grown using isotopically-depleted bacteria.

Figure 6. Partial FTMS *E. coli* JM-109 MALDI spectra over the range m/z 9140–9240. Full 2 M acquired data processed to obtained frequency domain spectrum. Cells grown using isotopically-depleted bacteria.

FTMS, broadband scans can now be used to identify eighteen ribosomal proteins and two DNA-binding proteins, including their post-translation modifications and associated water and ammonia losses, in a single highly resolved spectrum.

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