Anal. Chem. **2004,** *76,* 2103−2111

Hollow-Fiber Flow Field-Flow Fractionation for Whole Bacteria Analysis by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

Pierluigi Reschiglian,* Andrea Zattoni, Leonardo Cinque, and Barbara Roda

Department of Chemistry "G. Ciamician", University of Bologna, Via Selmi 2, I-40126 Bologna, Italy

Fabrizio Dal Piaz

Bioanalytical Mass Spectrometry Laboratory, Interdepartmental Center for Biotechnology Research, University of Bologna, Via Irnerio 46, I-40126 Bologna, Italy

Aldo Roda

Department of Pharmaceutical Sciences, University of Bologna, Via Belmeloro 6, 40126 Bologna, Italy

Myeong Hee Moon

Department of Chemistry, Yonsei University, Seoul 120-749, Korea

Byung Ryul Min

Department of Chemical Engineering, Yonsei University, Seoul 120-749, Korea

This work proposes for the first time the use of hollow-fiber flow field-flow fractionation (HF FlFFF) for improved matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOFMS) of whole bacteria. HF FlFFF has proved to be able to prepurify or fractionate different species of whole bacteria. Sample preparation by HF FlFFF gives improved spectra quality because noncellular components possibly present in the sample can be separated from the cells. When a mixture of two bacteria (*Bacillus subtilis* and *Escherichia coli*) is fractionated through HF FlFFF, MALDI/TOFMS analysis of each separated bacterial species preserves the most characteristic ion signals of the species without the presence of characteristic signals of the other species. The main advantages of HF FlFFF for MALDI/TOFMS analysis of whole bacteria are miniaturization, simplicity, and low cost of the fractionator components. This low cost makes disposable usage of the fractionator possible, thus eliminating the risk of run-to-run contamination of spectra due to sample carryover. The low fractionator volume yields bacterial fractionation on the order of a few minutes, which is comparable to MALDI/TOFMS analysis time. The small fractionation volume makes sample dilution low enough so that additional sample concentration steps are not strictly required to preserve MALDI/TOFMS detection.

Rapid, accurate identification of microorganisms is a key task in many areas, from medical to environmental studies. The increased threat of biological warfare and the strategies to counteract bioterrorism are the most recent, most widespread reminders of the urgent need to fulfill this task. Since 1975, mass spectrometry (MS) techniques have been proved to be powerful tools for the characterization of microorganisms.[1] Among them, matrix-assisted laser desorption/ionization time-of-flight MS (MALDI/TOFMS) analysis of intact microorganisms has become the most straightforward method to rapidly analyze intact microorganisms.[2,3] Fenselau and co-workers[4] can be credited with the broad development and application of the MALDI/TOFMS methods for the characterization of intact microorganisms, including instrumentation, sample treatments, and data handling.

It is widely recognized that the species desorbed from bacteria cells by MALDI and detected in TOFMS spectra are intact proteins in the M_r range 4000−15 000 and that most proteins coded by bacterial genomes fall within this molar mass range.[5] Biomarkers for different bacterial species can then be found in this range, and bacteria can be identified through proteomic database-searching algorithms.[6,7] These identification methods are robust

* Corresponding author. Fax: +39 051 209 9456. E-mail: pierluig.reschiglian@unibo.it.

(1) Anhalt, J. P.; Fenselau, C. *Anal. Chem.* **1975**, *47*, 219−225.
(2) Holland, R. D.; Wilkes, J. G.; Rafii, F.; Sutherland, J. B.; Persons, C. C.; Voorhees, K. J.; Lay, J. O. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 1227−1232.
(3) Jarman, K. H. *Anal. Chem.* **2000**, *72*, 1217−1223.
(4) Fenselau, C.; Demirev, P. A. *Mass Spectrom. Rev.* **2001**, *20*, 157−171.
(5) Demirev, P. A.; Ho, Y. P.; Ryzhov, V.; Fenselau, C. *Anal. Chem.* **1999**, *71*, 2732−2738.
(6) Demirev, P. A.; Lin, J. S.; Pineda, F. J.; Fenselau, C. *Anal. Chem.* **2001**, *73*, 4566−4573.

10.1021/ac0353379 CCC: $27.50
Published on Web 02/24/2004

vs spectral variability. Nonetheless, protein databases are as yet available only for a limited number of bacterial species. For this reason, the most common approaches so far employed to identify unknowns by MALDI/TOFMS analysis of intact bacteria are based on the similarity between the spectra of the unknown bacteria and those in MALDI/TOFMS libraries of reference bacterial species.[3] However, for these methods to be valid, a high degree of reproducibility is required. This is a particularly critical aspect in the identification of bacteria mixtures, because the resulting spectra are highly complex. Problems recently described in differentiating bacteria mixtures have focused on the need for very efficient MALDI/TOFMS data analysis algorithms.[8] Likewise, many experimental factors are also known to affect the quality and reproducibility of MALDI/TOFMS of whole bacterial cells.[4,5,9−11]

Accuracy and reproducibility of MALDI/TOFMS analysis of whole bacteria samples can be enhanced by coupling this physicochemical method to complementary techniques, as indicated in the literature.[4] Bioactive sample handling, which uses affinity surfaces for sample deposition, has recently been proposed.[12] Recently, it has been demonstrated that more accurate mass assignments are possible through high-resolution MS methods used together with MALDI/TOFMS.[13] Separation methods are, however, the most natural complements to increase the information obtained from MS analysis of complex samples. Incidentally, MALDI/TOFMS of bacteria mixtures is not only complicated by the high number of ion signals in the spectra, but also by the fact that MALDI is a competitive ionization process, and the spectra of bacteria mixtures can be quite different from the linear combination of characteristic signals obtained for each individual bacterial species. As a consequence, comparing the characteristic signals obtained from bacteria mixtures with the ion signal databases obtained with individual bacterial species could give inaccurate results. Sample preparation methods able to separate whole cells can potentially reduce the analytical complexity and difficulties in interpreting spectra obtained for bacteria mixtures by enriching the fractionated sample in one bacterial species.

Few separation methods are available for whole cell samples. Capillary electrophoresis has shown high performance in bacteria separation,[14,15] but differences in electrophoretic mobilities of bacteria show high variability because they depend on preparation of the cell dispersions. Over more than a decade, field-flow fractionation (FFF)[16,17] has proved its ability to fractionate and purify cells through a mechanism which is "soft" enough to preserve cell viability.[18] In 1991 a specifically designed variant of FFF proved able to select *Escherichia coli* mutants on the basis of motility differences.[19] Sedimentation field-flow fractionation (SdFFF) of flagellated and nonflagellated *E. coli* strains was then reported as a clever example of biological FFF applications.[16] Furthermore, differences in morphology indexes between different types of bacteria, as well as between live and dead bacteria of the same type, was proved to give different elution profiles in SdFFF and flow field-flow fractionation (FlFFF).[20] We have reported that both gravitational FFF (GrFFF) and flow FFF (FlFFF) are able to sort deactivated *E. coli* strains for whole-bacteria vaccines on the basis of differences in bacterial membrane features.[21]

Coupling FlFFF with MALDI/TOFMS of whole bacteria was recently presented.[22] That work addressed the problems related to biocompatibility and compatibility of the FlFFF mobile phase with the MALDI source, throwing light on three issues that could affect future use of FFF technology for MALDI/TOFMS of whole bacteria. First, possible run-to-run sample carry-over due to incomplete recovery in FFF could affect reproducibility of spectra and thus reduce the fingerprinting capabilities of MALDI/TOFMS. Second, the relatively high sample dilution after the FFF step could result in cell concentrations that are below the detection limits for MALDI/TOFMS. Third, the time required by the FFF step could affect the intrinsic rapidity of MALDI/TOFMS analysis.

Hollow-fiber FlFFF (HF FlFFF) is a trial prototype, a variant of FlFFF based on the idea of utilizing hollow fibers (HFs) of submillimeter diameter as cylindrical, microcolumn fractionation channels.[23] Early versions of HF FlFFF proved to be able to fractionate proteins and other water-soluble polymers.[24,25] More recently, a wider selection of HF membranes and improved HF FlFFF system technology have made it possible to fractionate synthetic organic-soluble polymers,[26] nano-sized[27,28] and micrometer-sized particles.[29] Performance of the most recent HF FlFFF version is comparable to that of commercial, macrocolumn FlFFF, the key advantages being reduced sample loads and dilution, shorter analysis time, and possible disposable usage of the channel. We have recently demonstrated that HF FlFFF is a reproducible method to fractionate different bacteria, such as deactivated *Vibrio cholerae* and *E. coli* strains for whole-cell bacterial vaccines as well as different types of human (red blood) and yeast (*Saccharomyces*

(7) Pineda, F. J.; Antoine, M. D.; Demirev, P. A.; Feldman, A. B.; Jackman, J.; Longenecker, M.; Lin, J. S. *Anal. Chem.* **2003**, *75*, 3817−3822.
(8) Wahl, K. L.; Wunschel, S. C.; Jarman, K. H.; Valentine, N. B.; Petersen, C. E.; Kingsley, M. T.; Zartolas, K. A.; Saenz, A. J. *Anal. Chem.* **2002**, *74*, 6191−6199.
(9) Williams, T. L. *J. Am. Soc. Mass Spectrom.* **2003**, *14*, 342−351.
(10) Wang, Z.; Russon, L.; Li, L.; Roser, D. C.; Long, S. R. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 456−464.
(11) Arnold, R. J.; Reilly, J. P. *Anal. Biochem.* **1999**, *269*, 105−112.
(12) Afonso, C.; Fenselau, C. *Anal. Chem.* **2003**, *75*, 694−697.
(13) Jones, J. J.; Stump, M. J.; Fleming, R. C.; Lay, J. O., Jr.; Wilkins, C. L. *Anal. Chem.* **2003**, *75*, 1340−1347.
(14) Armstrong, D. W.; Schulte, G.; Schneiderheinze, J. M.; Westenberg, D. J. *Anal. Chem.* **1999**, *71*, 5465−5469.
(15) Armstrong, D. W.; Schneiderheinze, J. M. *Anal. Chem.* **2000**, *72*, 4474−4476.
(16) Giddings, J. C. *Science* **1993**, *260*, 1456−1465.
(17) Giddings, J. C. *Field-Flow Fractionation Handbook*; Schimpf, M. E., Caldwell, K., Giddings, J. C., Eds.; Wiley-Interscience: New York, 2000; Chapter 1.
(18) Lucas, A.; Lepage, F.; Cardot, P. *Field-Flow Fractionation Handbook*; Schimpf, M. E., Caldwell, K., Giddings, J. C., Eds.; Wiley-Interscience: New York, 2000; Chapter 29.
(19) Berg, H. C.; Turner, L. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 8145−8148.
(20) Saenton, S.; Lee, H.; Gao, Y.-S.; Ranville, J. F.; Ratanathanawongs Williams, S. K. *Sep. Sci. Technol.* **2000**, *35*, 1761−1775.
(21) Reschiglian, P.; Zattoni, A.; Roda, B.; Casolari, S.; Moon, M. H.; Lee, J.; Jung, J.; Rodmalm, K.; Cenacchi, G. *Anal. Chem.* **2002**, *74*, 4895−4904.
(22) Lee, H.; Ratanathanawongs Williams, S. K.; Wahl, K. L.; Valentine, N. B. *Anal. Chem.* **2003**, *75*, 2746−2752.
(23) Lee, H.-L.; Reis, J. F. G.; Dohner, J.; Lightfoot, E. N. *AIChE J.* **1974**, *20*, 776−784.
(24) Wijnhoven, J. E. G. J.; Koorn, J.-P.; Poppe, H.; Kok, W. Th. *J. Chromatogr., A* **1995**, *699*, 119−129.
(25) Wijnhoven, J. E. G. J.; Koorn, J.-P.; Poppe, H.; Kok, W. Th. *J. Chromatogr., A* **1996**, *732*, 307−315.
(26) van Bruijnsvoort, M.; Kok, W. Th.; Tijssen, R. *Anal. Chem.* **2001**, *73*, 4736−4742.
(27) Lee, W. J.; Min, B.-R.; Moon, M. H. *Anal. Chem.* **1999**, *71*, 3446−3452.
(28) Moon, M. H.; Lee, K. H.; Min, B.-R. *J. Microcolumn Sep.* **1999**, *11*, 676−681.
(29) Min, B.-R.; Kim, S. J.; Ahn, K.-H.; Moon, M. H. *J. Chromatogr., A* **2002**, *950*, 175−182.

cerevisiae) cells.[30,31] The purpose of this paper is to show for the first time the applicability of HF FlFFF for MALDI/TOFMS analysis of whole bacteria. With respect to commercial FlFFF technology and related methodology, two key features of our HF FlFFF technology offer potential advantages for MALDI/TOFMS characterization of whole bacteria: (a) the low cost of the few parts that constitute the fractionator suggest that it may be possible to produce disposable units, thus reducing maintenance and problems of sterilization, eliminating the risk of run-to-run sample contamination; and (b) the reduced fractionator volume (typically 100−150 μL) reduces sample fractionation volume, which in turn reduces sample dilution. Such low sample dilution means that fewer concentration steps have to be performed on the fractionated samples to maintain MALDI/TOFMS detectability, thus decreasing total analysis time and simplifying possible system automation. Indeed, typical bacteria fractionation time through HF FlFFF is on the order of a few minutes; thus, it is comparable to the rapidity of MALDI/TOFMS characterization of bacteria, a key feature when rapid identification of pathogenic bacteria is required. It must, however, be noted that this HF FlFFF apparatus is still in the early stages of development and requires time-consuming, manual setup before analysis. Most likely, technical evolution and system automation will reduce the overall time required for the sample preparation step and make use of HF FlFFF for routine MALDI/TOFMS effective. In this work, we first describe the HF FlFFF selection of whole bacterial cells on the basis of differences in their physical features. With the mobile phase chosen for this study, we then demonstrate that HF FlFFF can improve MALDI/TOF spectra of cultivated, single bacteria strains through in-channel sample purification. Finally, we demonstrate that HF FlFFF can improve MALDI/TOFMS analysis of bacteria in mixture. The choice fell to a model mixture of *E. coli* and *Bacillus subtilis* in view of their HF FlFFF retention and MALDI/TOFMS characterization. The two bacteria are, in fact, both rod-shaped, but *B. subtilis* cells are ∼3−5 times longer (∼3−10 μm) than *E. coli* cells (∼1−2 μm). We then found different HF FlFFF retention of the two bacteria, because of the large differences in the cell size. Moreover, many examples of MALDI/TOFMS analyses of these two bacteria can be found in the literature,[4,5] the genomes of the two bacteria being thoroughly known and the related proteome further investigated. These features made evaluation of the results obtained by coupling HF FlFFF with MALDI/TOFMS most immediate. When the model mixture was separated through HF FlFFF, the MALDI/TOF spectra of the fractions corresponding to each separated bacterial species contained the spectral characteristics of the single species and no characteristic signals of the other species.

EXPERIMENTAL SECTION

1. Sample Preparation. Three bacterial species were used in this work. *B. subtilis* (ATCC 6633) and *E. coli* (ATCC 11303) were obtained as lyophilized cells from Sigma Aldrich (St. Louis, MO). *Bacillus clausii* spores were obtained from the pharmaceutical preparation Enterogermina (Sanofi-Synthélabo, Paris, France), which is a suspension of 2×10^9 spores/5 mL in sterilized water. Lyophilized cells were dispersed in mobile phase, then centrifuged, resuspended in the mobile phase, and allowed to equilibrate at least 6 h before the analysis. Cultivated cells were grown 16 h at 37 °C on LB agar plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 1.5% agar, pH 7.0, from Becton Dickinson and Company, Sparks, MD). When cultivated bacteria were analyzed, plated colonies were picked up from the agar plates, resuspended in 150 mM NaCl, and then centrifuged. Pellets were then resuspended in the mobile phase and centrifuged, and the resulting pellets were resuspended in the mobile phase before the analysis. *B. clausii* spores were directly injected as the original suspension or were diluted in the mobile phase. Approximately 10^5−10^6 cells were applied to the sample holder in a 1-μL droplet. When necessary, before MALDI/TOFMS analysis, the sample fractions collected from the HF FlFFF were concentrated 10-fold in a single centrifugation step.

2. HF FlFFF System. The disposable HF FlFFF fractionators were home-built as described elsewhere.[27−31] The HFs were made of polysulfone (PSf) with a 30 000 M_r cutoff, kindly supplied by SK Chemicals (Seoul, Korea). Channels were 24 cm in length with a 0.0410-cm inner diameter (dry conditions). Details on the home-developed HF FlFFF system were described in previous work.[31] Disposable usage of a channel typically involves three analysis steps. First a "makeup" HF FlFFF run is performed with an aliquot of the sample to condition the HF membrane of the new channel. Two further steps, which correspond to different flow rates and flow patterns, are then performed: (a) sample injection/focusing/relaxation and (b) channel elution. In step (b), one HPLC pump generates the required channel flow rates, while in step (a), a second, syringe pump is also used to inject/focus/relax the sample inside the channel. The HPLC pump was a model 422 (Bio-Tek Kontron Instruments, Milan, Italy), and the syringe pump was a model Pump 11 (Harvard Bioscience, Holliston, MA). Sample injection was made via a model 7125 injection valve (Rheodyne, Cotati, CA) equipped with an external 5.0-μL PEEK loop. The flow pattern conversions required to switch from step (a) to step (b) were achieved using four- and three-way switching valves. Flow rates were adjusted with the use of SS-SS2-VH Nupro metering valves (Nupro, Willoughby, OH), and the values were measured by burets and a chronometer. The flow pattern for step (a) was set before sample injection via a 3-way, T-valve (Hamilton, Reno, NV), as described in ref 31. Step (a) was run for 3−5 min, with the focusing point determined as previously described.[30] Step (b) was set by turning off the syringe pump used for step (a) with the HPLC pump set at the chosen elution flow rate value (V_{in}). Flow rate switching operations generated pressure pulses and variations of the mobile phase flow rate, and these were responsible for the transient signals often observed in correspondence with the fractogram void peak. The model UV 6000 LP (ThermoQuest, Austin, TX), high-sensitivity UV/vis diode-array detector equipped with a fiber-optic guide, 5-cm light-pipe cell was employed. The cell path length was measured with a spectroscopic standard, as described in a previous work.[30] The result was 4.6 ± 0.3 cm.

2.1. HF FlFFF Mobile Phase. Part of the experimental work addressed compatibility of the HF FlFFF mobile phase with whole bacteria MALDI/TOFMS. In fact, to minimize possible interactions between the HF channel inner wall and bacterial cells and,

(30) Reschiglian, P.; Roda, B.; Zattoni, A.; Min, B.-R.; Moon, M. H. *J. Sep. Sci.* **2002**, *25*, 490−498.

(31) Reschiglian, P.; Zattoni, A.; Roda, B.; Cinque, L.; Melucci, D.; Min, B.-R.; Moon, M. H. *J. Chromatogr., A* **2003**, *985*, 519−529.

thus, achieve the highest sample recovery, the mobile phase used in the HF FlFFF step must be a good dispersant. Moreover, it must also be biocompatible and must not suppress the ion signals of cells collected from the HF FlFFF outlet. A MALDI/TOFMS-compatible mobile phase for FlFFF of whole bacteria was recently described by other authors.[22] That work showed that adding the nonionic surfactant Triton X-100 to the 10^{-4} M NH_4Cl in the FlFFF mobile phase did not diminish the intensity of the MALDI/TOFMS signals. It is known that ammonium salts are volatile and do not produce stable adducts with proteins during ionization. However, it is also known that Triton X-100 can induce cell lysis to some extent. If cell lysis occurs during either sample preparation or the HF FlFFF run, reproducibility of MALDI/TOFMS analysis may be seriously compromised. In this work, the most appropriate mobile phase was found to be a solution of 1 mM ammonium cholate (Sigma Aldrich) in Milli-Q water (Millipore, Bedford, MA) at pH 9.2. Previous studies[31] have shown that because of their specific biocompatibility properties,[32,33] when added to the HF FlFFF mobile phase, bile acids were able to give high-recovery, reproducible HF FlFFF of cells such as red blood cells.

3. MALDI/TOFMS. For MALDI/TOFMS, typical procedures for the analysis of whole bacteria were adopted. On the MALDI target, 1 μL of bacterial dispersion containing $\sim 10^5-10^7$ cells was mixed with 1 μL of a solution of 1% (w/v) ferulic acid in 50% acetonitrile and 25% formic acid (Merck, Darmstadt, Germany). Such an acid matrix solution gave efficient bacterial lysis during the crystallization process and provided the most intense and reproducible ion signals. The MALDI/TOFMS instrument was a Voyager DE Pro (Applied Biosystems, Foster City, CA) equipped with a pulsed-N_2 laser operating at 337 nm. Positive ion spectra were acquired in linear mode over an m/z range from 4000 to 17 000 using a 20 000-V accelerating voltage, an 18 800-V grid voltage, and a delay extraction time of 250 ns. The final spectrum was obtained by summing the spectra acquired over five different spots. The spectrum for each spot was obtained by averaging the result of 100 laser shots. Mass calibration was performed as suggested in the literature for MALDI/TOFMS of *E. coli* and *B. subtilis*.[5]

RESULTS AND DISCUSSION

1. HF FlFFF. *1.1. Sorting by Cell Morphology.* The main objective of this paper is to demonstrate that HF FlFFF can be used as an instrumental, sample treatment step that is able to enhance MALDI/TOFMS methods for characterization of whole bacteria, particularly in the case of bacteria in a mixture. Discussion on the dependence of HF FlFFF retention on cell characteristics stands beyond the aims of this work and has been reported in our previous studies.[30,31] Cell retention is governed by the steric/hyperlayer mechanism,[34,35] under which it depends on differences in physical and membrane features of the cells.[18] HF FlFFF bacteria sorting can thus reduce sample complexity by reducing such cell differences between and within the fractionated subpopulations. Figure 1 reports two examples of HF FlFFF of bacterial species. Figure 1a shows that spores of *B. clausii* (fractogram 2) are more retained than lyophilized *E. coli* cells (fractogram 1). The *B. clausii* spores have, in fact, spherical shape, with size ($\sim 1-2$ μm) comparable to the length of the rod-shaped *E. coli* cells that have an aspect ratio of ~ 2.7.[21] Thus, the lower retention of the *E. coli* cells is a consequence of the higher aspect ratio. Figure 1b shows that the retention of *B. subtilis* cells lyophilized and suspended in the liquid medium is completely different from that of cultivated cells. This can be explained by the fact that the cell physical features depend on the cell growth stage.

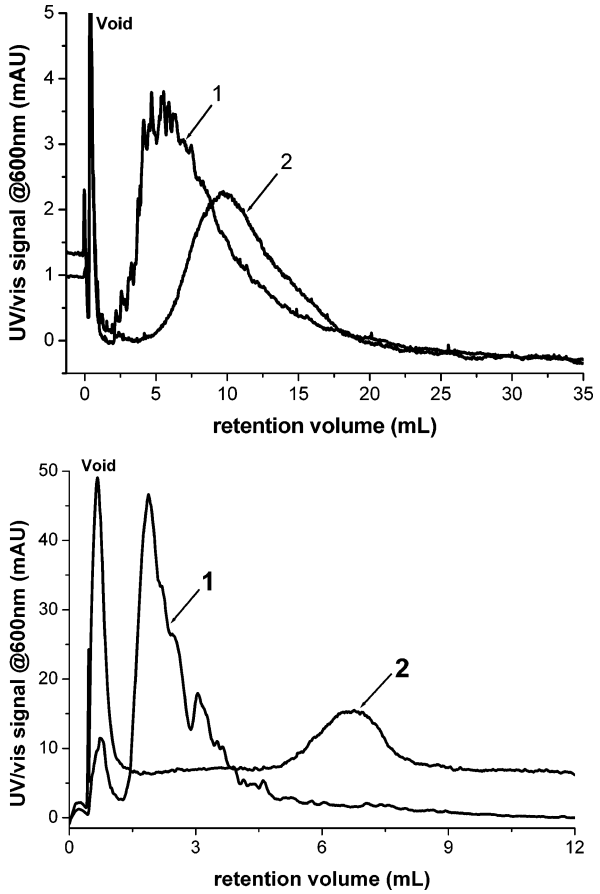

Figure 1. HF FlFFF of different bacteria. (a) Lyophilized *E. coli* (fractogram 1) cells, *B. clausii* spores (fractogram 2); $V_{in} = 3.0$ mL/min, $V_{rad} = 0.3$ mL/min. (b) Lyophilized *B. subtilis* (fractogram 1) cells, cultured *B. subtilis* (fractogram 2) cells; $V_{in} = 4.0$ mL/min, $V_{rad} = 0.7$ mL/min. Mobile phase: 1 mM ammonium cholate, pH = 9.2.

1.2. Analysis Time, Sample Dilution and Recovery. Handling time, dilution, recovery, and possible cross-contamination of the sample are important issues that must be addressed in choosing an effective sample treatment for MALDI/TOFMS. The rapidity with which MALDI/TOFMS methods identify whole bacteria should not be affected by long sample pretreatment steps. Because of the reduced HF FlFFF channel volume, HF FlFFF fractionation times on the order of a few minutes can be obtained, as shown in Figure 1a. Small fractionation volume gives low sample dilution. This makes it possible to obtain enough cells for MALDI/TOFMS analysis from fractions collected from just a single HF FlFFF run. This is the first key feature of HF FlFFF when compared to

(32) Roda, A.; Hofmann, H. F.; Mysels, K. J. *J. Biol. Chem.* **1993**, *258*, 6361−6370.
(33) Vadnere, M.; Lindenbaum, S. *J. Pharm. Sci.* **1992**, *71*, 881−883.
(34) Caldwell, K. D.; Cheng, Z. Q.; Hradecky, P.; Giddings, J. C. *Cell Biophys.* **1984**, *6*, 233−251.
(35) Caldwell, K. D. *Field-Flow Fractionation Handbook*; Schimpf, M. E., Caldwell, K. D., Giddings, J. C., Eds.; Wiley-Interscience: New York, 2000; Chapter 5.

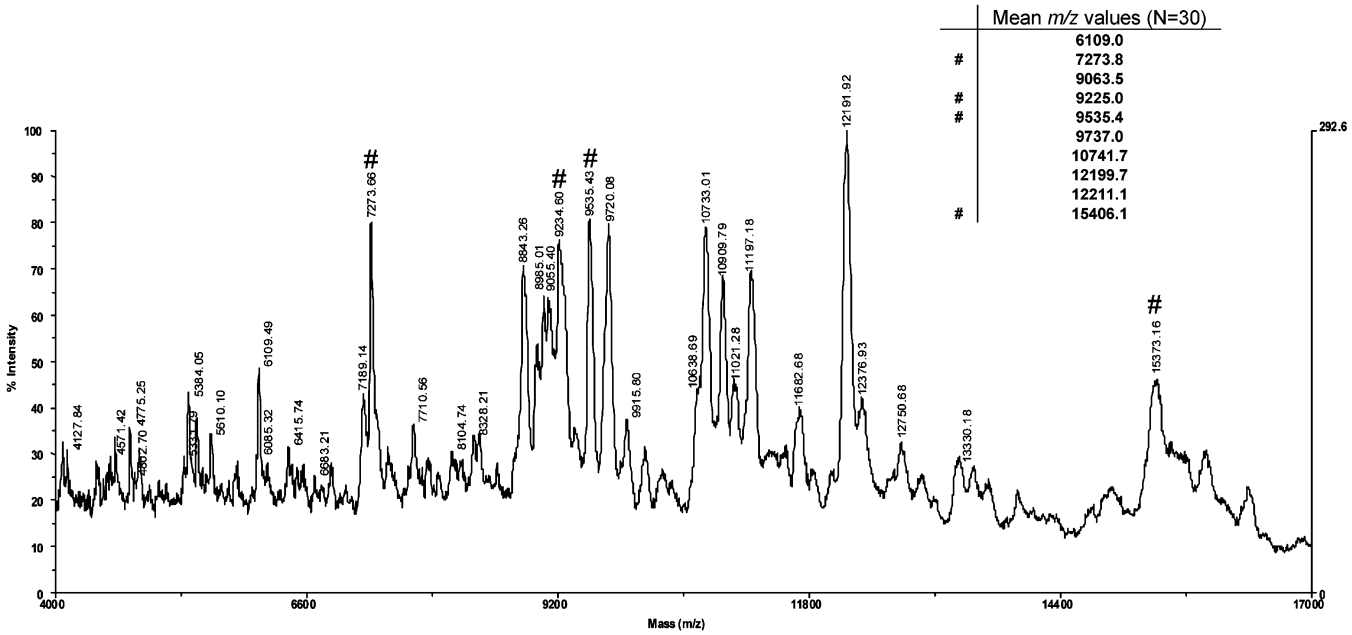

Figure 2. MALDI/TOF spectrum of cultured *E. coli* cells from a single colony. #, most characteristic *E. coli* peaks recovered.

commercial FlFFF technology, in which a relatively high sample dilution can result in fewer ion signals than in the case of nonfractionated samples.[22] Second, high sample recovery is another important feature in sample preparation for MALDI/TOFMS. In fact, after the fractionation step, incomplete sample recovery can affect MALDI/TOFMS sensitivity, reproducibility, and accuracy in identification. In particular, if absolute sample recovery is incomplete—that is, if part of the injected cells are trapped in the fractionator and thus remain uneluted—some sample components can be carried over to the next analysis. The use of disposable HF FlFFF channels intrinsically eliminates such a risk of run-to-run sample carry-over. However, even with disposable fractionators, high levels of proportionate recovery are necessary. In fact, proportionate recovery reflects the recovery of the different sample components in amounts proportional to their absolute amounts in the sample.[36] Therefore, in our case, high proportionate recovery means that the mass ratios between bacterial components of the sample are maintained after fractionation. With the mobile phase chosen in this work, 100% proportionate recovery was always found. Successful recovery of the different bacterial components in amounts proportional to their levels in the original sample makes a clear contribution to accurate and representative MALDI/TOFMS analysis of the sample fractions collected after HF FlFFF.

2. Coupling HF FlFFF and MALDI/TOFMS. Two databases of most characteristic ion signals of *E. coli* and *B. subtilis* were created by run-to-run and day-to-day replicates of MALDI/TOF spectra. For each bacterial species, the m/z obtained with a deviation of less than ± 5 m/z units between repeated MALDI/TOF spectra were taken. Ten so-determined, highly characteristic signals were found to be sufficient for our purposes. Lyophilized cells were used to eliminate possible sources of spectra irreproducibility, since the spectral profiles of a given isolate can be altered with the use of the same culture media from different manufacturers, different batches of culture media from the same manufacturer, or by the bacterial growth stage.[37] Table 1 reports the databases obtained in this manner. It is worth noting that a comparison of the MALDI/TOFMS signals obtained in Table 1 with *E. coli* identification data reported in the literature, performed by making a comparison between Fourier Transform MS and MALDI/TOFMS[13], and with data obtained from a model-derived protein biomarker search (http://infobacter.jhuapl.edu/)[7] makes it possible to assign at least one of the most characteristic ion signals found for *E. coli* to one *E. coli* protein: the 50S ribosomal unit L29, SwissProt #P02429 (m/z = 7273.8).

Table 1. Database of the Most Characteristic MALDI/TOFMS Signals Found with Run-to-Run and Day-to-Day Variations of Less Than ±5 *m/z* Units

E. coli mean m/z ($N = 30$)	*B. subtilis* mean m/z ($N = 22$)
6109.0	4260.3
7273.8	4445.1
9063.5	5638.2
9225.0	7328.0
9535.4	7369.9
9737.0	7538.1
10741.7	7549.8
12199.7	9078.6
12211.1	11255.2
15406.1	11262.9

2.1. In-Channel Sample Purification. Although HF FlFFF is mainly a separation technique able to sort bacterial cells according to differences in the cell physical features, an important aspect of HF FlFFF is its in-channel purification of bacterial samples before MALDI/TOFMS, because during the HF FlFFF run, the noncellular components can be removed or separated from the bacterial samples. First, the M_r cutoff of the HF membrane (30 000

(36) Ratanathanawongs-Williams, S. K.; Giddings, J. C. *Field-Flow Fractionation Handbook*; Schimpf, M. E., Caldwell, K., Giddings, J. C., Eds.; Wiley-Interscience: New York, 2000; Chapter 21.

(37) Du, Z.; Yang, R.; Guo, Z.; Song, Y.; Wang, J. *Anal. Chem.* **2002**, *74*, 5487−5491.

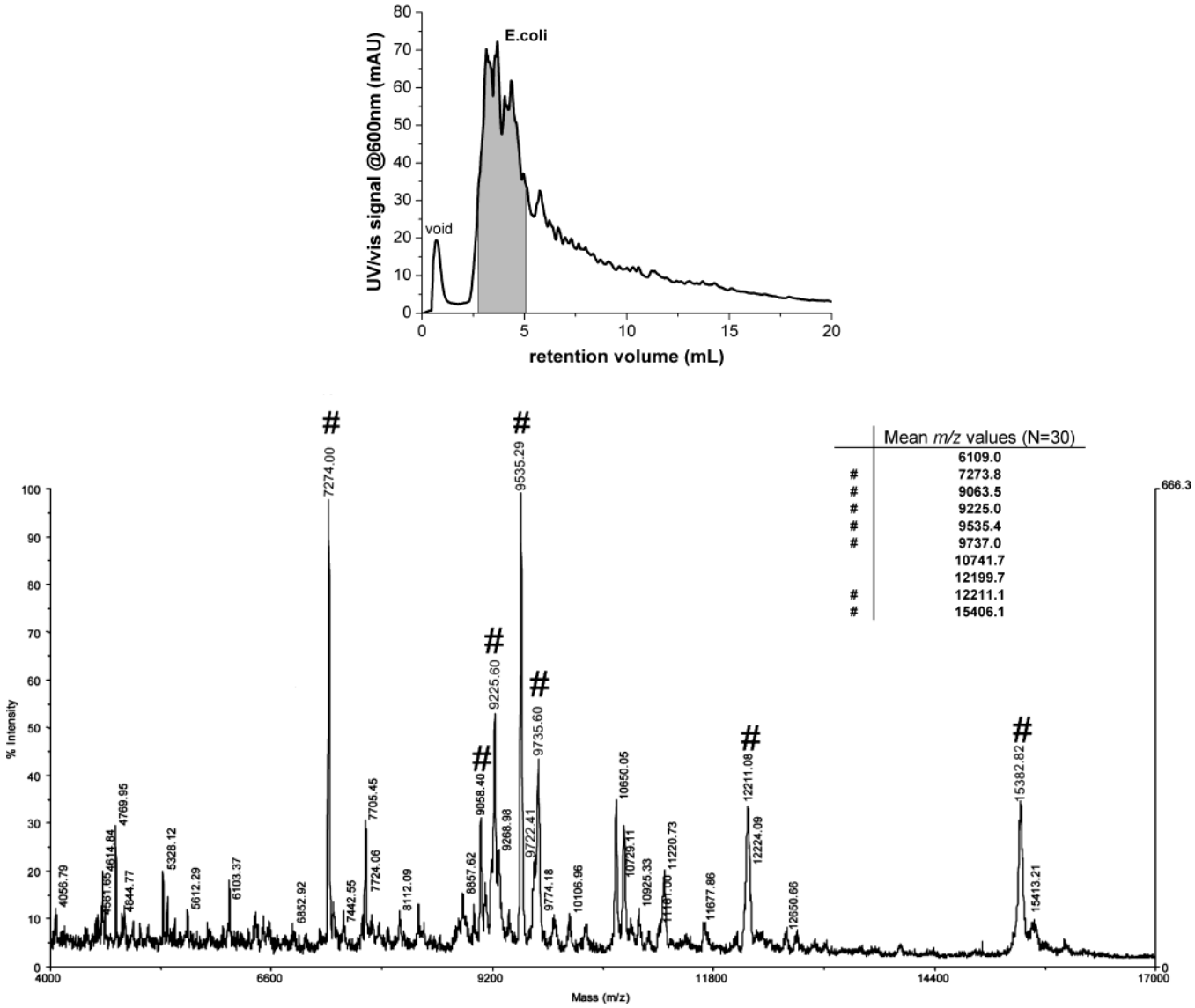

Figure 3. HF FlFFF MALDI/TOFMS of cultured *E. coli* cells. (a) HF FlFFF fractogram. V_{in} = 4.0 mL/min; V_{rad} = 0.7 mL/min. Mobile phase: 1 mM ammonium cholate, pH = 9.2. Fraction collected from 50 to 90 s; (b) MALDI/TOF *m*/*z* spectrum of the collected fraction. #, most characteristic *E. coli* peaks recovered.

in this work) ensures that noncellular components of molar mass lower than the HF cutoff (e.g., bacteria metabolites) are washed out through the HF pores, particularly during the focusing/relaxation step. Second, under the flow rate conditions employed here, noncellular components with molar mass higher than the HF cutoff (e.g., proteins in solution from the culture media, cell debris) elute either in correspondence to the void or at a shorter retention time than the bacterial cells. A particularly intense signal corresponding to the void is, in fact, observed in Figure 1b, fractogram 2, which corresponds to the fractionation of cultured cells. This makes HF FlFFF sample cleanup more efficient than filtration, since filtered samples would, indeed, retain noncellular components of M_r higher than the M_r filter cutoff.

Figure 2 shows the MALDI/TOF spectrum obtained from a single colony of cultivated *E. coli* without HF FlFFF treatment. Only 4 of the 10 *E. coli* ion signals listed in Table 1 can be assigned on the basis of the correspondence among the *m*/*z* values. Since the databases in Table 1 were obtained with lyophilized cells, the fact that fewer of the most characteristic ion signals are recovered in Figure 1 should confirm either that MALDI/TOF spectra profiles are dependent on the bacteria growth stage or that the components of the culture media can affect mass spectral profiles. It is known that ion signal broadening and reproducibility of MALDI/TOF spectra can be affected by the presence of noncellular sample components able to cocrystallize with the matrix.

Figure 3 reports the spectrum (Figure 3b) obtained after HF FlFFF (Figure 3a) of the clone of the same *E. coli* colony whose spectrum is reported in Figure 2. The observed fractionation volume is small, as obtained above also for other bacterial cells (see Figure 1a,b), and as previously found for HF FlFFF of other type of cells.[31] This small fractionation volume gave low sample dilution. The spectrum in Figure 3b was, in fact, obtained after one 10-fold concentration step of only one collected fraction (the fraction indicated in Figure 3a). A comparison with the spectrum in Figure 2, obtained with cultured cells, improves the information content in the spectrum of the collected fraction (Figure 3b). Seven

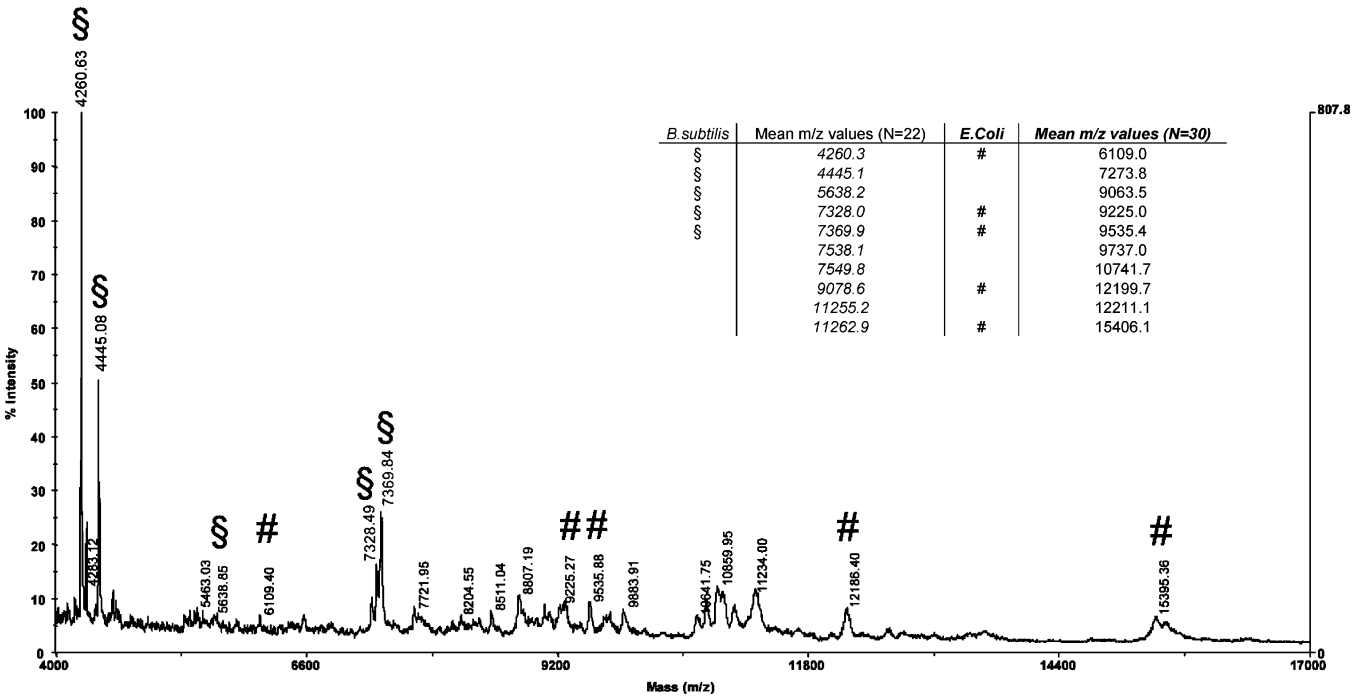

Figure 4. MALDI/TOF *m*/*z* spectrum of a 1:1 mixture of lyophilized *E. coli* and *B. subtilis* cells. #, most characteristic *E. coli* peaks recovered; §, most characteristic *B. subtilis* peaks recovered.

of the 10 most characteristic ion signals of the *E. coli* database (Table 1) can be assigned in Figure 3b, whereas only 4 were recognized when the colony was analyzed by MALDI/TOFMS without HF FlFFF (Figure 2). Of the seven most characteristic ion signals recovered in Figure 3b, the presence of the signal most likely assigned to the ribosomial protein SwissProt #P02429 is again confirmed. The increased quality of the spectrum in Figure 3b is not only evident in terms of number of most characteristic peaks. Ion signal intensities are also increased vs noise intensity, and signal broadening is reduced. Finally, the almost perfect correspondence between the spectrum in Figure 3b and the spectra of the lyophilized cells used for the database of the most characteristic *E. coli* ion peaks reported in Table 1 (spectra not shown) suggest that the fraction collected in correspondence with the HF FlFFF band in Figure 3a was most likely made up of pure cells in the mobile phase. It must be noted that in this study, only one fraction was collected, and this corresponded with almost the entire band of the fractionated cells. As a consequence, possible spectral differences either within the cells of the collected fraction or between the cells of the collected fraction and the cells eluted in the tail region of the fractogram were not considered. However, it must be noted that any noncellular component present in the sample would have been removed by HF FlFFF sample cleanup, and no MALDI/TOF spectral differences within and among fractions obtained from FlFFF bands of whole bacteria were reported in the literature.[22]

2.2 Bacteria Mixture Analysis. Figure 4 reports the MALDI/TOF spectrum obtained by mixing approximately equal amounts of lyophilized *E. coli* and *B. subtilis* cells. For both *E. coli* and *B. subtilis,* only five of the most characteristic ion signals in Table 1 can be recovered. Incidentally, the *E. coli* signal that was likely assigned to the ribosomial protein SwissProt #P02429 can no longer be recognized in the spectrum in Figure 4. In fact, the relevant ion signal might have been suppressed by the two most characteristic *B. subtilis* signals at $m/z = 7328.49$ and $m/z = 7369.84$. This finding would represent a significant loss of analytical information for possible identification purposes. In fact, the mass of protein #P02429 corresponds to the retention of the terminal methionine residue.[13] This protein chemistry is known to be relatively unusual in bacteria and can be considered consistent with the biology of *E. coli*.

Figure 5a reports the HF FlFFF fractogram obtained for the same 1:1 mixture of *E. coli* and *B. subtilis*, the MALDI/TOF spectrum of which is reported in Figure 4. Good separation can be observed between the two bands. By comparing the fractogram in Figure 5a with the fractograms obtained for each individual bacterial species (Figure 1a,b), each band in Figure 5a can be respectively ascribed to the elution of *B. subtilis* (band A) and *E. coli* (band B). If each band in the HF FlFFF fractogram in Figure 5a derives from the elution of just a single bacterial species, we could expect to obtain two completely different spectra from MALDI/TOFMS analysis of the fractions collected in correspondence to bands A and B, with the spectral features found for each individual bacterial species found in each spectrum. This is what it is shown in Figure 5b,c, which reports the MALDI/TOF spectra obtained for fractions 1 and 2, respectively, in Figure 5a. Eight of the 10 most characteristic *B. subtilis* ion signals and none of the 10 most characteristic *E. coli* ion signals in Table 1 are found in the spectrum of Figure 5b. When this spectrum was compared to the spectrum obtained after HF FlFFF of lyophilized *B. subtilis* cells (data not shown), the spectra profiles corresponded almost perfectly. Similarly to what observed for *B. subtilis*, in Figure 5c, 9 of the 10 most characteristic *E. coli* ion signals and none of the 10 most characteristic *B. subtilis* ion signals in Table 1 are found. It is also worth noting that when the spectrum in Figure 5c is superimposed on the spectrum in Figure 3b (obtained after HF FlFFF of cultured *E. coli* cells), the correspondence is almost perfect.

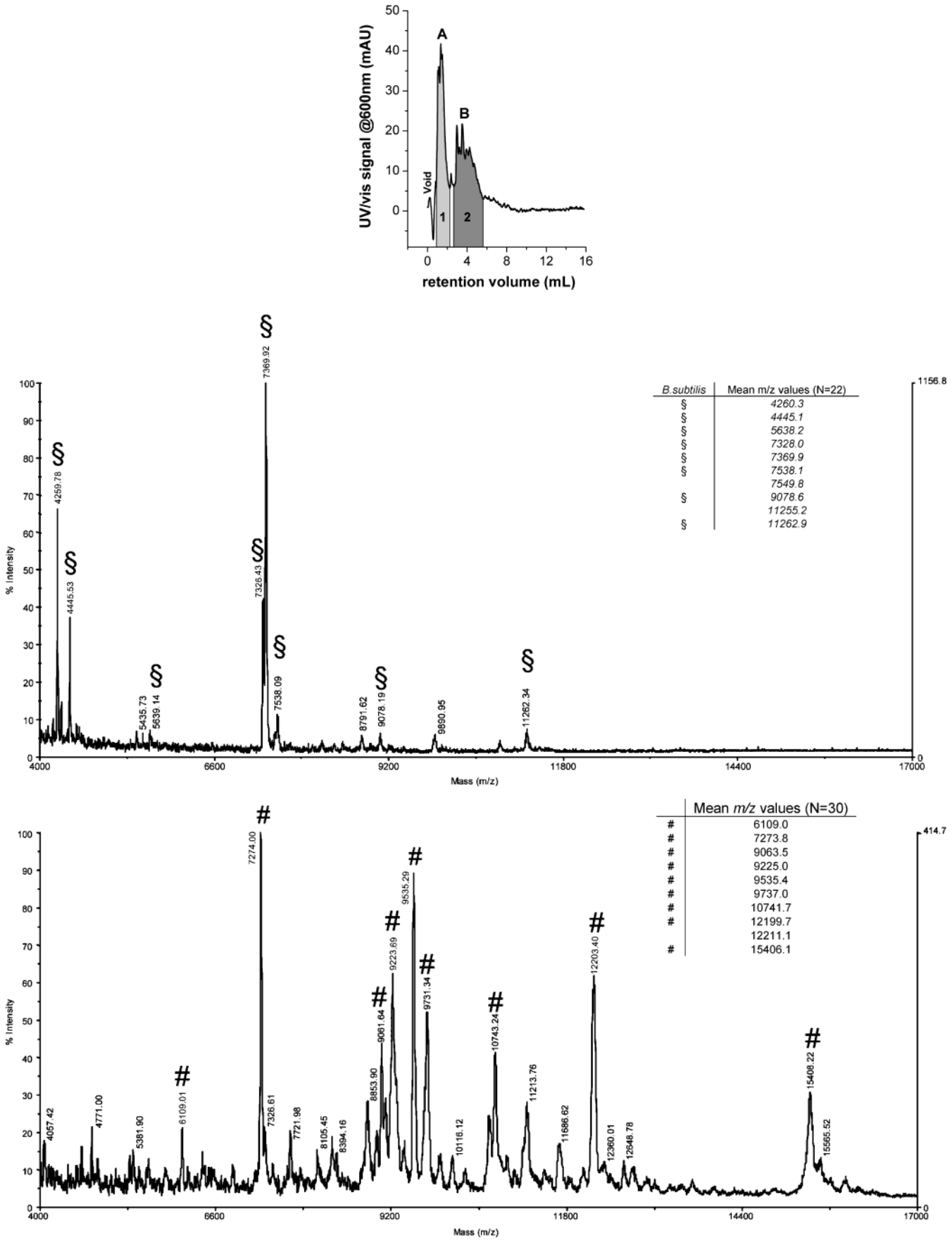

Figure 5. HF FlFFF MALDI/TOFMS of a 1:1 mixture of lyophilized *E. coli* and *B. subtilis* cells. (a) HF FlFFF fractogram. V_{in} = 4.0 mL/min; V_{rad} = 0.8 mL/min. Mobile phase: 1 mM ammonium cholate, pH = 9.2. Peak A, *B. subtilis*; Peak B, *E. coli*. Fraction collection times: fraction 1 (*B. subtilis*) from 20 to 40 s; fraction 2 (*E. coli*) from 50 to 100 s. (b) MALDI/TOF *m*/*z* spectrum of the collected fraction 1; §, most characteristic *B. subtilis* peaks recovered. (c) MALDI/TOF *m*/*z* spectrum of the collected fraction 2; #, most characteristic *E. coli* peaks recovered.

All these findings confirm, first, that total separation of *B. subtilis* from *E. coli* was obtained by means of HF FlFFF. A powerful identification technique such as MALDI/TOFMS can thus evaluate HF FlFFF separation performance for mixtures of whole bacteria. Second, comparison between the *E. coli* spectra in Figures 5c and 3b also indicates that, when HF FlFFF is employed as a pre-MS step, no significant differences in the MALDI/TOF spectral features appear between lyophilized (Figure 5c) and cultured *E. coli* cells (Figure 3b). This provides additional evidence that the spectral differences observed when cultured *E. coli* cells were analyzed in MALDI/TOFMS with (Figure 3b) or without (Figure 2) the HF FlFFF pre-MS step could be mostly due to the presence of extracellular components in the sample, rather than to differences in the bacteria growth stage.

Finally, it is worth noting that even the *E. coli* ion signal most likely assigned to the protein #P02429 is recovered in the spectrum from fraction 2 (Figure 5c), but it was lost in the spectrum for the 1:1 *E. coli* + *B. subtilis* mixture obtained without HF FlFFF (Figure 4). This indicates that when MALDI/TOFMS identification of *E. coli* is to be performed in samples in which bacteria other than *E. coli* may be present, the use of HF FlFFF provides significantly greater analytical information.

CONCLUSIONS

This work shows that HF FlFFF can be effectively coupled with MALDI/TOFMS to improve spectral analysis of samples of whole bacteria. The HF FlFFF cleanup/fractionation step improves spectral quality, since noncellular components possibly present in the sample can affect accuracy and reproducibility of MALDI/TOFMS analysis. While evident spectral differences appear whether cultured cells are run through HF FlFFF or not, no spectral differences between lyophilized and cultured cells appear after HF FlFFF of the cultured cells. These findings suggest that HF FlFFF may be used to reduce spectral variability when bacterial identification is to be performed by MALDI/TOFMS fingerprinting.

Improvements in MALDI/TOFMS analysis through HF FlFFF are particularly evident in the case of bacteria in mixture. In the future, simple, disposable fractionator units could be used in automated HF FlFFF systems to improve MALDI/TOFMS methods for characterizing complex bacterial samples. Such a technology should have interesting applications in various areas, from biohazard monitoring to, for instance, characterization of whole-bacteria vaccines and other products derived by biotechnological processes. However, it must be mentioned that the total HF FlFFF separation of the two bacteria species (*E. coli* and *B. subtilis*) in the model mixture used here was actually chosen to stress feasibility of HF FlFFF for MALDI/TOFMS analysis of bacteria mixtures. It is, in fact, an extreme case. Instead of a "chromatography-like" separation between two populations of cells with very different size, such as *E. coli* and *B. subtilis*, HF FlFFF fractionation would most frequently involve the enrichment of cells with some given characteristics vs the entire population. This is because, in FFF, cell sorting is generally the result of a continuous distribution of multipolydispersity in different cell physical features. The high fractionation capabilities of HF FlFFF then elute the sample cells as relatively broad bands, because the multipolydispersity in the different cell physical features is translated into a continuous difference in cell retention. As a consequence, total HF FlFFF separation between bacterial species in bacteria mixtures is not necessary to make future, effective use of HF FlFFF for MALDI/TOFMS of intact bacteria. HF FlFFF band-slicing before MALDI/TOFMS should be enough to decrease the complexity of cell mixtures and to adequately enrich the HF FlFFF fractions in a specific bacterial species that, thanks to this enrichment, could subsequently be better identified by MALDI/TOFMS. To increase sorting performance and, thus, increase sample enrichment vs a particular cellular species, tandem HF FlFFF (HF FlFFF^n) could be performed by the reinjection of the band slice for further runs through HF FlFFF. Because of in-channel sample focusing before each HF FlFFF step, HF FlFFF^n is not expected to significantly increase the final sample dilution and affect MALDI/TOFMS detectability of the fractionated cells. Since HF FlFFF proves able to sort bacteria of a given species according to differences in the cell physical features,[30,31] HF FlFFF^n may also assist MALDI/TOFMS in characterizing subpopulations of a given bacterial species. Work is in progress to develop HF FlFFF^n methodology for simplified and more automated bacterial sample preparation for MALDI/TOFMS analysis.

ACKNOWLEDGMENT

The authors are thankful to the Interdepartmental Center for Research in Biotechnology, University of Bologna (Italy), and to the Center of Applied Biomedical Research, S. Orsola Hospital, Bologna, for the MALDI/TOFMS instrumentation. Thanks also goes to Sonia Casolari, Dora Melucci, Marco Dell'Erba, and Daniela Parisi, Department of Chemistry "*G. Ciamician*", Bologna, for their technical assistance and experimental work on the HF FlFFF system; to Massimo Guardigli and Mara Mirasoli, Department of Pharmaceutical Sciences, Bologna, for their technical assistance on MALDI/TOFMS measurements and bacteria cultivation; and to Eileen Nan Cartoon for English revision. Grants: Work supported in part by the Italian Ministry of the Education, University and Research (MIUR, COFIN 2000, MM03247343-005); and by the University of Bologna, funds for selected research topics.

Received for review November 12, 2003. Accepted January 21, 2004.

AC0353379