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Investigating the effects of protein patterns on microorganism identification by high-performance liquid chromatography–mass spectrometry and protein database searches

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Abstract

High-performance liquid chromatography–electrospray ionization mass spectrometry (HPLC–ESI-MS) has been employed for separation and detection of protein biomarkers from *E. coli* samples. LC–MS is suitable for microbial identification because it can couple on-line with sample clean-up devices and is readily amenable to automation. In this work, we have investigated the effects of sample preparation methods on the detection of bacterial proteins by LC–MS. Many factors effect the degree of variations in the protein patterns (i.e. number and masses of proteins). For example, changing the polarity as well as pH of the extraction solvent may control the number of detected proteins. It is also noted that the protein patterns can vary even when the total ion chromatography plots seem to be the same under the same sample preparation conditions. Further, we have tested experimentally the influence of LC–MS-analyzed protein patterns (molecular masses between 2000 and 60,000) on microbial identification by protein database searches. This is in contrast to the current database search approach, where only the masses of smaller proteins (≤20,000) from direct matrix-assisted laser/desorption ionization MS are used. In spite of the variations in protein patterns, all the database search results show that the best matches come from the correct microorganism.

Keywords: *Escherichia coli*; Protein database searches; Proteins

1. Introduction

Analysis and identification of protein profiles in cells and tissues is an important area in biological research [1]. One of the applications is to identify microorganisms, which is critical in medical microbiology and in detecting biohazards in the environment [2]. The number of proteins present in a microorganism can be large and their abundances may vary in several orders of magnitude. For example, the estimated total number of *Escherichia coli* proteins derived from protein analysis experiments as well as open reading frame is more than 5000 [3]. Among such a large pool of proteins, several proteins may be used as biomarkers for bacterial identification. Recent technical advances in mass spectrometric ionization and detection methods have provided a way to characterize biopolymers. Both matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) mass spectrometry (MS) can be used for protein analysis and therefore, for bacterial identification. At the present time, MALDI-MS is generally used to obtain mass spectra of proteins from bacterial samples [4–15].

A successful identification of bacteria by protein

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profiling using MALDI-MS is based on a high level of mass spectral reproducibility. However, the spectra of such complex systems depend on many experimental conditions and microbiological factors. For example, (a) the selection of matrixes; (b) the sample-matrix preparation methods; (c) bacterial sample pretreatment methods; (d) bacterial growth times. The experiments need to be carefully controlled in order to get good reproducibility [9,16,17]. Moreover, MALDI-MS is relatively difficult to couple on-line with sample pretreatment and separation techniques and is not readily amenable to automation. This limitation poses a major challenge in some real world sample analyses, where on-line detection and automation are major concerns.

ESI-MS analyzes ions that are produced directly at atmospheric pressure from liquid samples. Therefore, the method enables on-line combination with sample clean up, concentration and separation techniques such as microdialysis, solid-phase extraction, liquid chromatography, and capillary electrophoresis [18,19]. Liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) has been used to obtain reproducible protein profiles from bacterial samples [20]. The analysis of bacterial samples by the off-line coupling between LC and MS has also been reported [21,22]. Vaidyanathan et al. have recently reported a direct ESI-MS analysis of whole bacterial cells without prior separation [23]. Since ESI-MS is sensitive to buffer, salts and detergent [24], combination of separation methods and ESI-MS may give more reproducible spectral profiles of bacterial samples. Nevertheless, the sample pretreatment methods may still contribute to the spectral variations in the analysis of individual bacteria.

In the 1990s, the increase in biological information and the ease of sharing this information through the Internet led to a new scientific discipline, bioinformatics. Currently, more than 70 genomes of microorganisms are completely sequenced [25] and deposited in the publicly available databases. By using bioinformatics algorithms, the complete proteins of microorganisms with known DNA sequences can be established and are also accessible through the Internet. Fenselau's group proposed an approach for microorganism identification by mass spectrometry and protein database searches [26]. The mass spectral peaks were compared with the proteomes in the database. Probable organism sources were assigned to each peak and the best match (i.e. with most matched peaks) should lead to the identification of a right microorganism. For this approach, the relative peak intensities in the mass spectrum are not critical. It is the protein pattern (masses) that is the key to the success in microbial identification. Because of the overlap in masses of some proteins in different microorganisms, false identification can occur especially when the mass accuracy is low or the size of the proteome is large. If the reliability of database search results is deemed uncertain, a significance level testing as proposed by Fenselau and co-workers may be performed to evaluate the significance of the results [27,28].

Theoretically, the database search approach is independent of experimental conditions. The protein patterns may change and still give the correct identification of microorganisms. So far, this approach is based on the MALDI mass spectra of bacterial samples. The masses of proteins analyzed by direct MALDI-MS are mostly below 20,000 [4–15]. By using LC–MS, more proteins can be observed so can those proteins with higher mass. It would be interesting to test the effects of LC–MS data on the database search results. In this work, we have investigated the effects of several sample extraction solvents and extraction methods on protein patterns. Further, we have examined the effects of protein patterns on bacterial identification when a database search approach is used.

2. Experimental

2.1. Bacterial sample preparations

The *Escherichia coli* XL1 was grown in nutrient broth at room temperature for 24 h. Cells were harvested, washed with water three times, lyophilized to dryness and stored at −20 °C. The solvents used for protein extraction from the cells include water, 0.1% trifluoroacetic acid (TFA), 10 m*M* phosphate-buffered saline (PBS) and acetonitrile–water (40:60). The lyophilized bacteria were suspended in the solvents at a concentration of 5 mg/ml. The cell suspension was treated by different methods. These methods were vortexing, sonication

and freeze–thaw. For the vortexing method, several treatment times were used. For the liquid nitrogen freeze–thaw method, five cycles were employed to break the cells. The cell suspension was centrifuged and the supernatant was collected and filtered by a 4.5 μm poly(vinylidene difluoride) (PVDF) membrane. Unless noted otherwise, 500 μl of the supernatant solution were desalted three cycles and concentrated to 50 μl by Microcon YM-3 with a molecular mass cut-off of 3000 (Amicon, Oakville, Ontario). For the sample without desalting, 500 μl of supernatant solution were concentrated to 50 μl by SpeedVac. All the chemicals used above were purchased from Sigma.

2.2. LC–MS analysis

To analyze the samples by LC–MS, an ion trap mass spectrometer (LCQ Duo, Finnigan, San Jose, CA, USA) equipped with an electrospray ionization source was coupled to a 600E HPLC pumping system (Waters, Milford, CA, USA). The mass spectrometer was operated under the control of an Xcaliber program. In each experiment, 5 μl of protein extract were injected. Spectra were collected in the positive ion mode. The autogain control (AGC) was maintained at 1×10^7, with microscan count of 2 and maximum injection time of 100 ms. The spray voltage was maintained at 3 kV. The capillary voltage and temperature were kept at 27 V and 200 °C, respectively. The instrument was calibrated externally to achieve a mass accuracy of ±3 for cytochrome *c* and myoglobin.

The LC separation was carried out at a flow-rate of 0.2 ml/min on a Zorbox 3-μm C_8 column (150× 2.1 mm, Agilent Technologies). The gradient was developed over 30 min from 30 to 90% B. Mobile phase A consisted of 0.1% TFA in acetonitrile–water (5:95, v/v) and mobile phase B consisted of 0.085% TFA in acetonitrile–water (95:5, v/v). The acetonitrile was HPLC grade from J.T. Baker and the TFA was from Riedel-de Haën. The water was purified by Milli-Q (Millipore, Bedford, MA, USA). Gradient times were minimized to achieve rapid separations while still offering appropriate resolution to separate the most abundant bacterial proteins. All of the column eluent was diverted directly into the mass spectrometer. The molecular masses of separated proteins were determined by deconvolution of the ESI mass spectra using the Finnigan BIOMASS software.

2.3. Protein database searches

A search by protein molecular mass was performed in the SWISS-PROT or SWISS-PROT/TrEMBL database (Expasy, Swiss Bioinformatics Institute) using the Sequence Retrieval System. The Alternative Query form provides a number of classifiers for database search. We used averaged protein molecular mass as the primary classifier. The molecular mass window of ±3 was selected and ''bacteria'' was the only restriction applied in the query of protein search. The search result for each entry of a molecular mass (±3) gave the protein identities and organism sources. For the purpose of bacterial identification, only the probable organism sources were assigned for each protein mass obtained from LC–MS experiments.

3. Results and discussion

3.1. LC–MS analysis

The bacterial protein patterns obtained by mass spectrometry may be very different when various experimental conditions are used. Those conditions include sample extraction solvents, extraction methods and many others. We focus on most of the sample pretreatment methods that are LC–MS compatible and can potentially be coupled on-line with LC–MS. Fig. 1 shows the total-ion current-chromatogram (TIC) of protein extract using water as a solvent. The extraction time is 5 min under vortex mixing. From the TIC plot, about 20 peaks at most are observed. However, analyzing the mass spectra can identify 43 proteins. This is an advantage of using a mass analyzer over using a UV detector. Fig. 2 shows two mass spectra of bacterial proteins from the separation shown in Fig. 1. These two spectra are from the same total ion current (TIC) peak but are acquired during different periods of time near 22.2 and 22.4 min. The spectra clearly indicate two well-separated proteins even though the TIC plot shows only one peak.

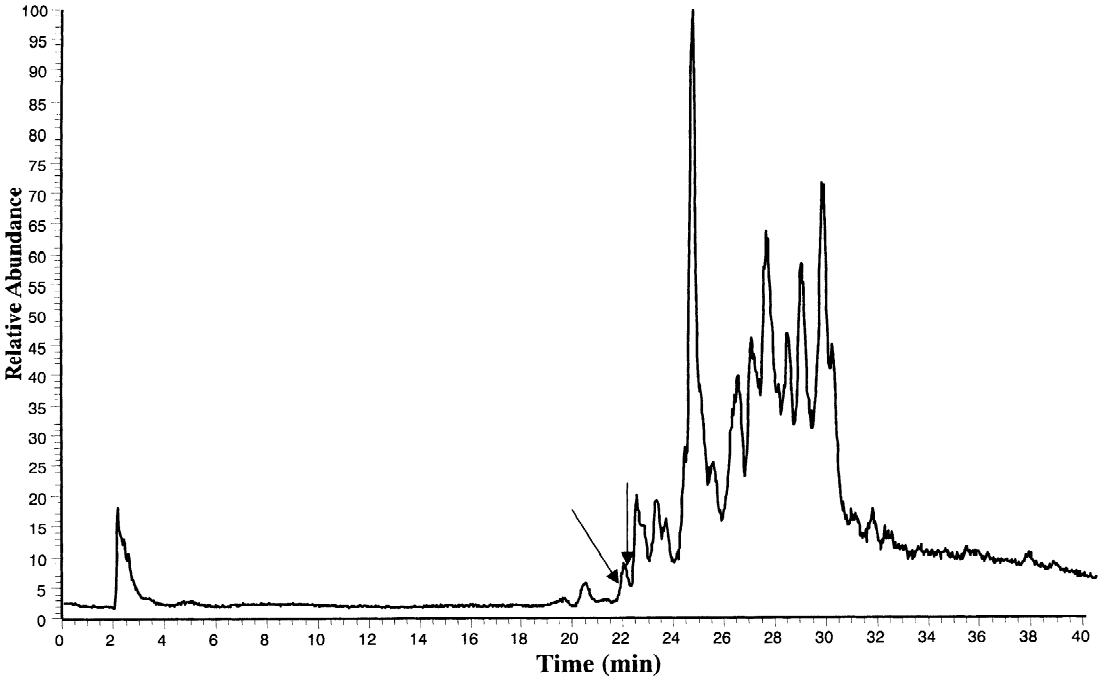

Fig. 1. TIC plot from the *E. coli* sample, using a vortexing method (5 min) and using water as the solvent.

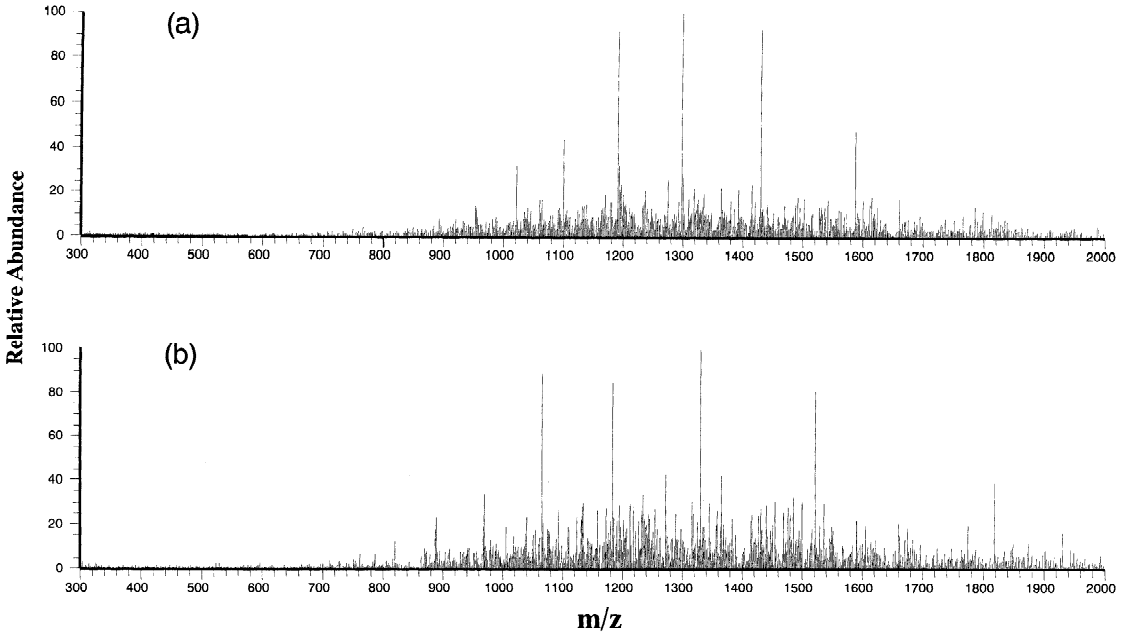

Fig. 2. Mass spectra acquired from the separation (indicated by arrows) shown in Fig. 1 during the periods between (a) 22.24–22.27 min and (b) 22.42–22.45 min.

Table 1
Masses of proteins observed from the separation shown in Fig. 1

2430	9739	12,230	18,123	33,418
6414	9982	13,482	18,159	33,725
7272	10,298	14,066	18,160	38,205
7706	10,385	14,286	18,568	40,715
8324	10,633	14,361	22,282	40,996
9123	10,692	15,952	23,005	45,369
9459	11,193	16,880	24,979	52,776
9548	11,989	17,053	28,510	
9573	12,214	17,552	32,374	

The masses of the observed proteins are listed in Table 1. For the *E. coli* sample, one might expect thousands of proteins to be detected. In fact, it is unlikely to separate so many proteins by using a single column. This is not only because those proteins have a wide range of isoelectric points (p*I*) but also because many proteins exist in very low abundance. The wide range of p*I* values limits the number of water-soluble proteins and the low abundance proteins are relatively hard to detect. To separate such a large number of proteins generally requires a two-dimensional gel technique. Recently, multidimensional HPLC involving the use of ion-exchange and reverse phase columns has been reported to separate proteins [29]. For the purpose of bacterial identification, detection of no more than 50 proteins was pursued in this work.

3.2. Effects of various sample preparation methods

The numbers and patterns of bacterial proteins obtained when using different solvents and different extraction methods were compared. Table 2 summarizes the numbers of proteins observed and the similarities between different protein patterns. There are 43 proteins that could be separated and identified when the sample in 0.1% TFA was vortexed for 5 min. The same number of proteins obtained from the water and 0.1% TFA experiments seems to be a coincidence. When the protein masses were compared, 24 proteins are identical within the mass accuracy. The pH of 0.1% TFA is about 2.0, while the pH of water is about 7.0. The solubilities of bacterial proteins in these two solvents should be very different. Therefore, it is not surprising that two experiments gave very different protein patterns. The PBS buffer (pH ~8.5) is found to give the greatest number of proteins (51). There are 31 proteins found in common between the water and PBS experiments, while the number of proteins found in both of the PBS and 0.1% TFA results is only 17. PBS and 0.1% TFA have the most different pH values among the solvents used and therefore, gave the most different protein extracts.

There are 15 proteins detected when acetonitrile–water (40:60) was employed as the extraction solvent. It is the least number of proteins obtained.

Table 2
Number of proteins and similarities between protein patterns obtained by using different sample preparation methods

Sample preparation	Number of observed proteins	Number of proteins in common
(a) Solvents (5 min vortexing)		
Water	43	–
0.1% TFA	43	24[b]
PBS	51	31[b]
Acetonitrile–water (40:60)	15	13[b]
(b) Extraction methods (in water)		
Vortexing[a]	43	–
Sonication	38	34[c]
Freeze–thaw	38	32[c]
(c) Salt contents		
PBS[a] (with membrane desalting)	51	–
PBS (without membrane desalting)	38	34[d]

[a] Experimental results are taken from (a) to make comparisons easy.
[b] Number of proteins that were also found in the water result.
[c] Number of proteins that were also found in the vortexing result.
[d] Number of proteins that were also found in the PBS (with membrane desalting) result.

Among those proteins detected, 13 proteins are also found in the water extraction experiment. The decrease of protein number may be attributable to the polarity decrease in this solvent. When an even less polar solvent such as pure acetonitrile was used, there was no protein detected. However, this does not mean that none of the bacterial proteins was soluble in acetonitrile. The acetonitrile-soluble proteins may exist in such abundance that they were not detected with the present experimental set-up.

Several groups have reported mass spectrometric analyses of bacterial samples by different protein extraction methods [4,6,7]. We have compared several simple extraction methods. The numbers of proteins and the similarities between different sets of proteins observed when using vortexing, sonication and freeze–thaw extraction methods are listed in Table 2. The water suspensions of bacterial samples were vortexed or sonicated for 5 min or under five freeze–thaw cycles before centrifugation. The numbers of proteins appear to be similar and the similarities between protein sets are high. Over all, there are more than 30 proteins in common among the three protein extracts.

ESI-MS is sensitive to buffers used in the sample solutions. The buffers can deteriorate the quality of mass spectra by suppressing the signals or complicating the mass peaks with adduct peaks. The comparison between protein patterns obtained with and without the use of a desalting membrane is made (Table 2). Much less proteins (38) are observed without using the desalting membrane. For comparison, the same LC gradient programs were used for the two runs. Although an LC separation should be able to desalt the sample, the presence of the buffer does have a significant effect on the signal detection by LC–MS. When analyzing the TIC for the sample without being desalted, some mass peaks disappear and some are too complicated to be mass-analyzed due to the salt adduct peaks. We believe that the incomplete desalting was due to the choice of the gradient (from 30 to 90% B).

The effect of extraction times on number of proteins obtained using the vortexing method was studied. The numbers of proteins obtained from the aqueous sample solutions with extraction times of 30 s, 2 min, 5 min and 30 min are 37, 36, 43 and 39, respectively. The extraction times for *E. coli* sample do not have a significant effect on the protein extraction. The similarity among the protein patterns is high (about 80% of proteins are in common). It is noted that the optimal extraction time may depend on bacterial samples. The present results indicate that proteins can be observed without extensive extraction, which is suitable for a fast bacterial analysis. The time-independence also reflects that the proteins observed are those that are most readily soluble in the solvent.

3.3. Reproducibility

We have demonstrated that protein patterns may change when different sample pretreatment methods are used. The protein maps can be different from sample to sample, even the TIC plots seem to be the same. The TIC plots of protein extracts (Fig. 3) were acquired over 2 weeks from the same batch of bacteria by vortexing the sample in water for 5 min. These TIC plots appear to be reproducible by visual inspection but detailed analysis of the mass spectra shows variations on those TIC plots (Table 3). This is partially because of the large number of proteins in a bacterial cell. Several proteins can be coeluted from time to time in the time scale used in this work so that the analysis of some protein masses becomes difficult. When we compared these results with the one shown in Table 2 (43 proteins detected), a larger difference was observed. This may be due to small variations in bacterial culturing conditions.

3.4. Protein database searches

When the bacterial identification is based on mass spectral fingerprints of bacterial samples, the accuracy relies on a good spectral reproducibility. In view of the effect of many experimental parameters on the observed fingerprints, the database searching approach seems to be an excellent alternative for bacterial identification. We compare the set of protein molecular masses obtained from LC–MS spectra of a bacterial sample against a database containing the molecular masses of proteins present in known organisms. Currently, there are more than 50,000 protein sequences of prokaryotic proteins derived from genomic open reading frame and nongenomic entries [3]. Therefore, it is expected that each

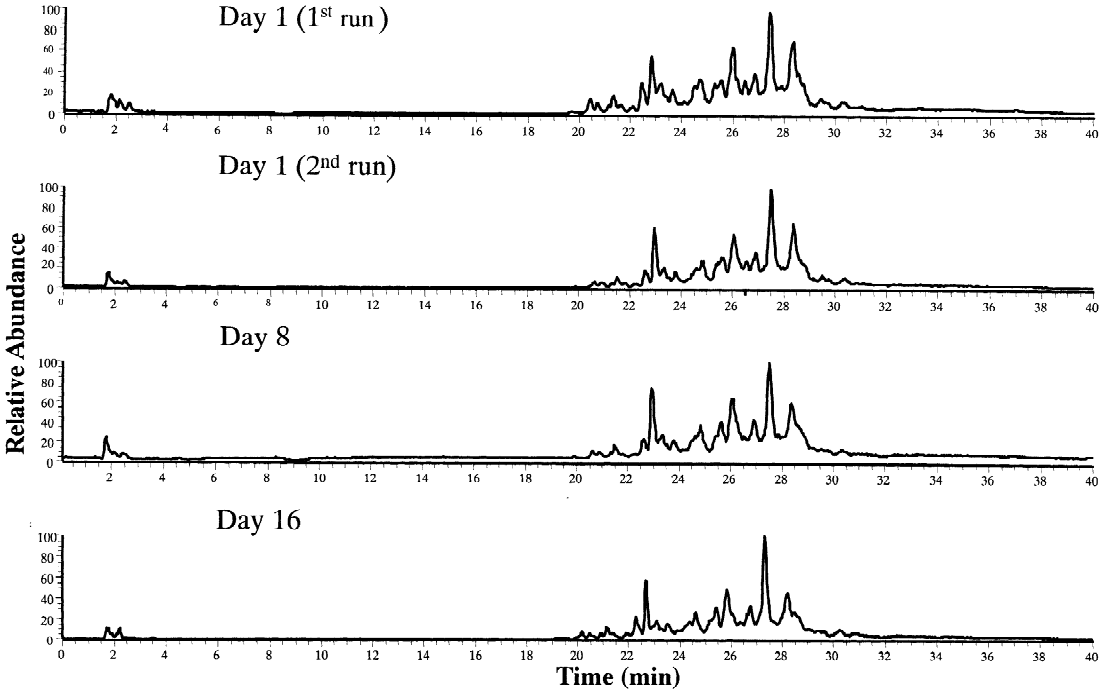

Fig. 3. TIC plots acquired over 2 weeks from protein extracts by using a vortexing method (5 min) and using water as the extraction solvent.

Table 3
Numbers of proteins and similarities between protein patterns obtained from the reproducibility experiments shown in Fig. 3

	Number of proteins	Number[a] of proteins in common
Day 1 (the 1st run)	36	–
Day 1 (the 2nd run)	37	31
Day 8	36	30
Day 16	38	32

[a] Number of proteins that were also found in the day 1 (the 1st run) result.

observed protein mass will match protein masses of many microorganisms.

To identify the microorganism by database searches, we assigned a matching rate to each microorganism. The microorganism with the highest matching rate is the most probable candidate. The matching rate is defined as the percentage of detected proteins that match the protein of each of the microorganisms in the database. The microorganisms with the highest and the 2nd highest matching rates based on the results using different sample treatment methods are summarized in Table 4. When the *E. coli* proteins are extracted by vortex mixing the

Table 4
Effects of sample preparation methods on the protein database search results

	Microorganism[a]	
	1st	2nd
Water (5 min vortexing)	*E. coli* (70)	*H. influenzae* (18)
0.1% TFA	*E. coli* (60)	*H. influenzae* (16)
Acetonitrile–water (40:60)	*E. coli* (80)	*B. subtilis* (27)
PBS (desalted)	*E. coli* (61)	*H. influenzae* (20)
Sonication	*E. coli* (58)	*H. influenzae* (18)
Freeze–thaw	*E. coli* (61)	*B. subtilis* (18)
PBS (nondesalted)	*E. coli* (66)	*B. subtilis* (26)

[a] Matching rates (%) are indicated in the parentheses.

water suspension, 30 out of 43 observed proteins match the proteins of *E. coli* in the SWISS-PROT database, giving a matching rate of 70%. The unmatched peaks for *E. coli* may be due to the alkali cation attachment, post-translational modification of the proteins, or the incomplete proteome. The matching rate is much higher than that of the 2nd matched microorganism (18% for *Haemophilus influenzae*). We want to point out that the matching rate is also dependent upon which database is used. For example, if the SWISS-PROT/TrEMBL database is used, the highest matching rate is 84% for *E. coli* while the matching rate is 55% for the 2nd matched microorganism, *Streptomyces coelicolor*. SWISS-PROT and TrEMBL are two different types of protein sequence databases. The former is an annotated sequence database where information is extracted from the literature. The latter is a repository of sequence data, which are translated directly from DNA sequences. Thus, searching the combination of two databases or the SWISS-PROT alone certainly gave different results. We focus on the searches in the annotated SWISS-PROT database.

From Table 4, the search results for the experiments using different sample preparation conditions all lead to the right bacterial source, *E. coli*. When using PBS as an extraction solvent, more matched peaks (31 peaks) were found but the matching rate is comparable (61%). Acetonitrile–water (40:60) gave a higher matching rate of 80%. To decide if less polar solvent is more useful in bacterial detection needs more data from different microorganisms. The results do indicate that we can potentially vary the experimental condition to achieve a more reliable identification of microorganism. The search results from desalted and nondesalted samples are compared. Although the nondesalted sample gave less protein signals, the matching rate (66%) is higher than that for desalted sample (61%). We also compare the database search results for the reproducibility experiments (data not shown). All the matching rates (around 60%) indicate *E. coli* as the organism source even though the experimental data show some variations in protein patterns. Table 5 shows the matching rates when only the protein molecular masses above 20,000 were used for database searches. The protein masses were obtained from the solvent effect study as shown in Table 2. The matching rates approximately parallel those obtained from the whole mass peaks.

Table 5
Effects of high masses (20,000–60,000) on the database search results

Solvent (5 min vortexing)	Microorganism[a]	
	1st	2nd
Water	*E. coli* (67)	*B. subtilis* (33)
0.1% TFA	*E. coli* (54)	*B. subtilis* (31)
Acetonitrile–water (40:60)	*E. coli* (100)	*B. subtilis* (50)
PBS (desalted)	*E. coli* (55)	*H. influenzae* (20)

[a] Matching rates (%) are indicated in the parentheses.

It is noted that false identification can occur when a large number of detected proteins match the masses of proteins in the database for an unrelated microorganism. The possibility of false identification increases if the mass tolerance is increased or if the size of the known proteome increases. The proteome of *E. coli* in the SWISS-PROT has 4305 proteins in the molecular mass range from 2000 to 60,000, which is a relatively large proteome. This means that the detected proteins are relatively more likely to match the protein masses of *E. coli* in the database. Strictly speaking, it requires a statistical analysis to evaluate the significance of search results. The false identification rate (significance level) that is a function of proteome size and mass accuracy can be calculated to indicate the significance. Applying the ''significance level'' to database searches is beyond the scope of the present work. Our purpose is to test the effects of different protein patterns obtained from the same bacterial source on the matched protein numbers. All of the database search results based on different experiment conditions gave relatively high matching rates. The results demonstrate that experimental parameters such as extraction solvents or extraction methods will not hamper the microbial identification when using database searches.

4. Conclusions

We have investigated the effects of sample preparation methods on the protein patterns obtained from *E. coli* samples. Most of the sample treatment methods tested are MS-friendly and may have potential for on-line coupling. Changing the polarity as

well as pH of the extraction solvent may control the protein patterns. When the proteins are extracted by using a buffer solution, the protein components detected by MS with or without a sample desalting step are quite different, considering LC is used for the protein separation. For the reproducibility experiment, the protein patterns can vary, while the TIC plots seem to be reproducible. This is partially because of the large number of proteins in a bacterial cell.

The variation of protein patterns underscores the importance of microbial identification by the protein database search approach. In principle, any combination of protein peaks is useful in the identification of microorganisms as long as the protein masses are characteristic of the microorganism. The effects of protein patterns obtained by different sample preparation methods on the percentages of matched proteins have been demonstrated. The wide mass range of bacterial proteins obtained by LC–MS gave relatively high matching percentages for the positive identification. Further, the high-mass range (20,000–60,000) gave matching percentages parallel with those obtained from the whole mass range (2000–60,000). In spite of the variations in protein patterns, all the database search results find the best match for the right microorganism. Future work will focus on finding the optimal sample preparation conditions for the general identification of microorganisms based on database searches.

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