Analysis of the *Salmonella typhimurium* Proteome using Fractionated LC-MS

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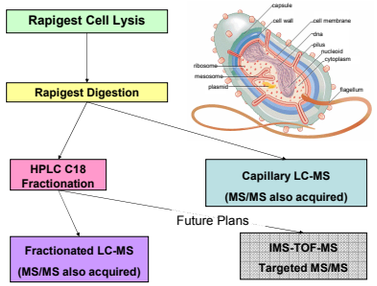
Overview

- The *Salmonella typhimurium* proteome was analyzed by "bottom-up" proteomic methods using both on-line capillary liquid chromatography mass spectrometry (LC-MS) and off-line LC fractionation MS
- Some proteomic experiments such as Targeted-MS/MS could benefit from direct infusion electrospray ionization (ESI)-MS method that allows extended analysis time.
- Capillary LC and LC fractionation methods were compared to evaluate the impact of fractionation on the accurate mass and elution time (AMT) tag approach for peptide identifications.

Introduction

The LC-MS coupled with ESI has proven to be a powerful tool for the study of global proteomes. LC has a number of attractive features including reducing the complexity of samples being infused into a mass spectrometer and the reproducible nature of LC can also be used as a determinant in the "identification" of peptides[1].

Despite the utility of LC, there are some limitations. One common limitation when performing LC-MS/MS is that a peak must be sampled prior to its fragmentation. Since many peaks may be eluting simultaneously for complex sample, it is often impossible to perform MS/MS analyses on all of the co-eluting species. The quality of the fragmentation spectrum depends also on when an initial detection of a precursor mass is made in an elution profile. We present here an alternative method that uses off-line LC fractionation in combination with automated sample infusion of each fraction using a Nanomate™ (Advion, New York) system. A peptide mixture from a tryptic digest of *Salmonella enterica* serovar Typhimurium (aka, *S. typhimurium*) was analyzed using this method and the performance of this off-line method was further compared with the on-line LC-MS/MS. *S. typhimurium* is a facultative intracellular pathogen that causes ~7,000 reported cases of acute gastroenteritis per year in the USA.



Proteomic sample preparation
This diagram summarizes the steps used to prepare the samples and how the same sample was characterized by different methods. The Rapigest™ cell lysis attempts to maintain representation from the various membranes and structures of the *Salmonella* cell (inset).

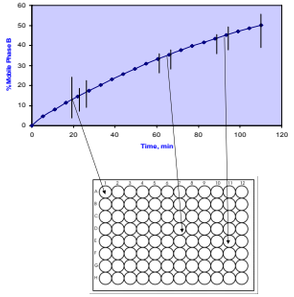
Methods

Salmonella typhimurium Cell Growth and Harvesting

Stationary phase cultures of *Salmonella enterica* serovar Typhimurium LT2 laboratory strain were used in all experiments[2].

Proteomic Sample Preparation

Cell pellets of *S. typhimurium* were lysed and digested using the Rapigest™ (Waters, Milford, MA) protocol, except trifluoroacetic acid (TFA) was substituted for hydrochloric acid for surfactant hydrolysis. Peptides were fractionated on an Agilent 1100 HPLC system with a C18 Jupiter (Phenomenex, Torrance, CA) column (2.1 x 250 mm, 5 µm, 300 A). The gradient started at 100% mobile phase A (0.2% acetic acid/0.5% TFA in water) and exponentially decreased to ~50% mobile phase A with mobile phase B (90:9.9:0.1 ACN:water:TFA) over 110 minutes, then changed to 100% mobile phase B for 10 more minutes. 80 fractions were collected from 20 to 120 minutes in a 96-well plate.



HPLC fractionation and collection
Traditional HPLC was performed on *S. typhimurium* peptides with a gradient designed to match our capillary LC-MS conditions. The analysis was fractionated in to 80 fractions and collected on a 96-well plate

Capillary LC-MS/MS

LC-MS/MS analysis was performed using a Thermo Electron model LTQ-Orbitrap mass spectrometer (Thermo Electron Corp., San Jose, CA) with electrospray ionization (ESI). The capillary LC column was coupled to the mass spectrometer with an in-house manufactured interface[3].

The heated capillary temperature and spray voltage were 200ºC and 2.2 kV, respectively. Data acquisition began 20 minutes after sample injection and continued for 100 minutes at AGC settings of 3x104 and 1x106 for the ion trap and Orbitrap, respectively. Orbitrap spectra were collected from 400-2000 m/z at a resolution of 60,000 followed by data dependant ion trap MS/MS spectra of the three most abundant ions using a collision energy setting of 35%. A dynamic exclusion time of 60 seconds was used to discriminate against previously analyzed ions.

Fractionated LC-MS/MS

Each of the 80 fractions was infused into an LTQ Orbitrap operated as described above for 5 minutes using NanoMate® from Advion Bioscience.

Data Analysis

SEQUEST Analysis

The MS/MS spectra were correlated with possible peptide identifications by using the SEQUEST algorithm to search the annotated *Salmonella typhimurium* database of proteins from TIGR (http://www.tigr.org/).

Peptide identification was based upon previously described criteria[4].

AMT Tag Approach

The Accurate Mass and Time approach[5] (AMT) was used to analyze MS results. For fractionated LC-MS/MS data two different strategies for AMT Tag Database construction were utilized.

In the first approach, a separate reference Mass and Time tag database was generated using only a small subset of LC-MS/MS analyses and the fractionated LC-MS/MS as described elewhere[5] with estimated elution times.

As part of a different project a reference Mass and Time tag database was generated from LC-MS/MS spectra from approximately 500 LC-MS/MS datasets[2].

A file containing mass and estimated elution times was assembled to allow peak matching with our publicly available informatic tool known as VIPER to allow peak matching between this file and a database.

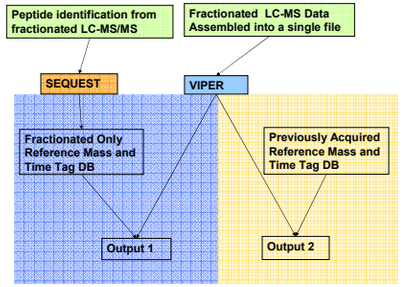
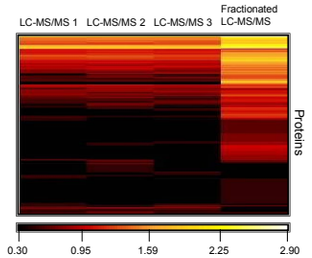
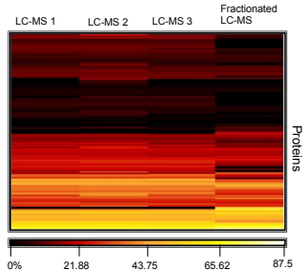


Diagram describing the two approaches in data analysis.
The results from Output 1 should have excellent agreement with elution times, but the LC resolution is by the nature of sample poor. Therefore, we demonstrated some limited utility with our traditional AMT tag approach with our production *Salmonella typhimurium* reference database.

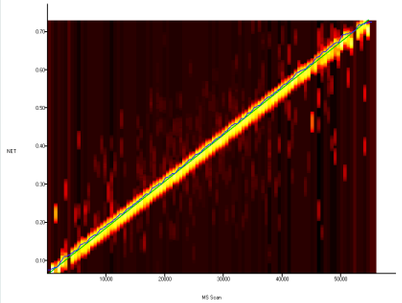
Results



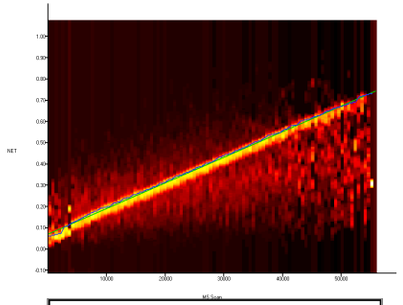
Peptide Counts Per Protein (logarithmic scale)
For each of the proteins detected by MS/MS (logarithmic scale). Only proteins that have more than two peptide counts are shown. Higher values of peptide counts for more abundant protein species in Fractionated LC approach.



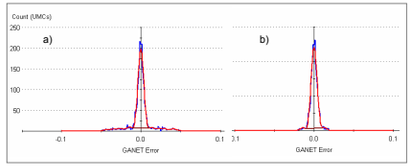
Heat Map of Protein Amino Acid Coverage (percent scale) Data shown for all proteins detected across 3 traditional LC-MS replicates and Fractionated LC-MS. Similar levels coverage were seen for most proteins between for the AMT approach.



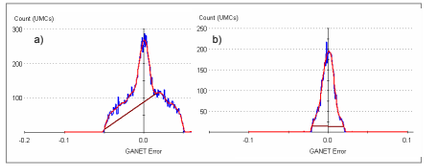
Elution time alignment of reference database using the LCMSWARP algorithm[6] - Output 1 (see Methods)



Elution time alignment of reference database using the LCMSWARP algorithm[6] – Output 2 (see methods)



Histogram of Normalized Elution Time (NET) Distribution for Output 1 (Fractionated reference database). Plot a) demonstrates NET distribution before LCMSWARP refinement and plot b) demonstrates distribution after the LCMSWARP alignment. Due to the constrained nature of the fractionating the distributions are nearly identical.



Histogram of Normalized Elution Time (NET) Distribution for Output 2 (LC-MS/MS base reference database). Plot a) demonstrates NET distribution before LCMSWARP refinement and plot b) demonstrates distribution after the LCMSWARP alignment. Due to the loss of LC resolution during fractionation is it expected that a poorer distribution of elution time would occur during the AMT tag approach.

Conclusions

- Fractionating a reversed phase LC separation could be used to overcome undersampling in LC-MS/MS at the cost of throughput. The reduction in complexity and the ability to perform at least a rudimentary AMT tag approach suggests LC-fractionation can be used with orthogonal separations of peptide to increase overall coverage.
- Since this method tends to perform a better sampling of the higher abundance species versus lower abundance species we have attained a more representative concentration standard for Salmonella proteins.

Acknowledgements

This research was supported by the National Institute for Allergy and Infectious Diseases (IA Y1-AI-4894-01). Work was performed in Environmental Molecular Sciences Laboratory at Pacific Northwest National Laboratory, a multiprogram national laboratory operated by Battelle for the U.S. Department of Energy under Contract No. DE-AC05-76RLO 1830.

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