# Application of label-free quantitative LC-MS-based proteomics for biomarker identification in *Salmonella* Typhimurium

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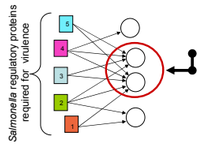
## Overview

- *Salmonella* infection is a major public health threat with ~1.4 million cases of infection annually at an estimated cost of ~$3 billion/year in the U.S. alone
- With the recent emergence of antibiotic-resistant isolates, an understanding of the pathogenesis of *Salmonella* is important for developing new therapeutic agents
- Knock-out mutants of 15 *Salmonella* regulators critical to systemic infections were developed, and the resulting perturbations relative to the wild-type proteome were analyzed using quantitative label-free LC-MS-based proteomics measurements to gain an understanding of the systems involved in virulence of *Salmonella*
- We identified a set of proteins coordinately regulated by a preponderance of the *Salmonella* regulators examined and up-regulated upon infection of host macrophage cells, providing novel targets for therapeutics

## Introduction

Non-typhoidal salmonellae (NTS) are common causal agents of acute food-borne disease worldwide, with the bacterium *Salmonella* Typhimurium a leading cause. In the U.S. alone there are ~1.4 million cases of *Salmonella* infections annually at an estimated cost of ~$3 billion/year (CDC - 07/08). Because of the increasing prevalence of antibiotic-resistant isolates, an understanding of the growth and pathogenesis of NTS, specifically *Salmonella* Typhimurium, is important for developing new therapeutic agents. As such, we applied quantitative label-free LC-MS-based proteomics measurements to gain an understanding of the systems involved in virulence of *Salmonella* Typhimurium.

We hypothesized that proteins coordinately regulated by *Salmonella* regulatory proteins required for virulence will represent the best targets for therapeutics (inset figure). As a first step in understanding how virulence is regulated, we constructed in-frame non-polar deletions of 84 regulators thought to play a role in *Salmonella* Typhimurium virulence and tested them for virulence. Proteomics profiling was carried out for each of the 15 most attenuated mutants under host-free culture conditions to identify proteins coordinately regulated by *Salmonella* regulatory proteins.



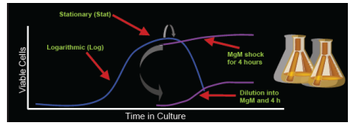

**Commonly regulated proteins represent best targets for therapeutics**

Our proteomics analysis has revealed a set of proteins that are coordinately regulated by a preponderance of the *Salmonella* regulatory proteins examined, co-regulated with known *Salmonella* virulence factors, and whose expression is up-regulated upon infection of host macrophage cells. These observations have been validated by Western Blot analysis. Taken together our data suggest this set of proteins play a central role in *Salmonella* infection of host cells, providing novel targets for therapeutics.

## Methods

### Virulence screen

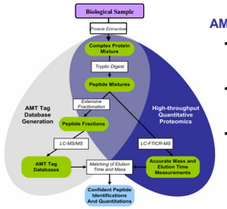
- In-frame non-polar deletions of 84 regulators constructed using method of Datsenko and Warner (*PNAS* 2000, *97*, 6640-6645), in which the entire coding region of a gene is replaced with a selectable antibiotic resistance gene (kan)
- Functional disruption of mutant derivatives confirmed by PCR analysis
- BALB/c mice i.p. infected with ~200 cfu of either regulator deletion mutant strain or wildtype strain




*Salmonella* Typhimurium grown in host-free culture conditions

- Laboratory conditions
  - Growth in Luria Bertani broth (LB) to log phase (Log)
  - Growth in LB to stationary phase (Stat)
- Intracellular environment mimic
  - Stat phase cells transferred to acidic minimal media for 4 h, then harvested (MgM Shock)
  - Stat phase cells diluted 1:100 into acidic minimal media growth for 4 h, then harvested (MgM Dilution)

### AMT tag approach: High throughput proteomics methodology

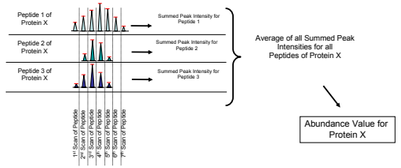



**AMT tag database generation**

- Complex samples fractionated, analyzed by LC-MS/MS, and spectra analyzed using SEQUEST
- Contains MS/MS peptide sequences, normalized elution times, and calculated masses
- Serves as "look-up table" for subsequent high throughput LC-MS analyses

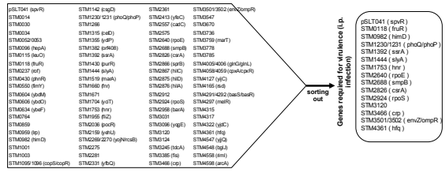
**High throughput quantitative proteomics**

- Peptides identified from LC-MS peaks by matching both measured accurate masses and normalized elution times to those in AMT tag database within specified values
- Protein abundance value calculation:



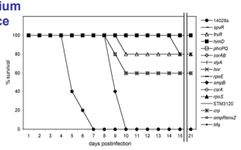

## Results

### Screening for regulatory proteins required for systemic infection



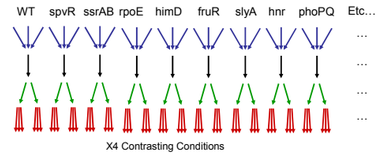

**Attenuated virulence of *S. Typhimurium* 15 regulatory mutants in BALB/c mice**

- BALB/c mice i.p. infected with ~ 200 cfu of *Salmonella* strain and observed for 21 days
- Percentage surviving mice shown for each strain



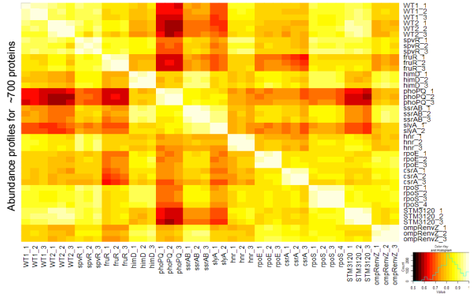

### Label-free LC-MS analysis

**Replicate analysis to account for variation**




- Samples blocked and randomized prior to analysis by LC-LTQ-Orbitrap
- Each LC-LTQ-Orbitrap replicate analysis has a strong correlation to the other two replicate analyses for each sample, indicating reproducibility between replicate runs

**Correlation plot of variation across MgM dilution growth condition**




### Generating a candidate list

**Significantly down-regulated proteins under MgM dilution growth condition (intra-macrophage like)**

| Protein | Frequency | Peptides | SpvR | FruR | HimD | PhoP/Q | SsrA/B | SlyA | Hnr | RpoE | CsrA | RpoS | STM3120 | OmpR/EnvZ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| STM0082 | 8 | 4 | -4 | NSD | -11 | -11 | -12 | NSD | NSD | -11 | -11 | NSD | -11 | |
| STM0831 | 8 | 8 | -2 | -3 | -10 | -3 | -2 | NSD | NSD | NSD | -13 | -4 | -6 | |
| STM1165 | 8 | 4 | NSD | NSD | -9 | -9 | -9 | -9 | -9 | -11 | NSD | -11 | NSD | -11 |
| STM1231 | 7 | 9 | NSD | NSD | -9 | -11 | -9 | -7 | -2 | -5 | NSD | NSD | NSD | -3 |
| STM1469 | 10 | 3 | NSD | -10 | -10 | -10 | -7 | -10 | -10 | -7 | -7 | -5 | NSD | -7 |
| STM1548 | 7 | 7 | NSD | NSD | -10 | -10 | -11 | NSD | NSD | -12 | -12 | -12 | NSD | -12 |
| STM1599 | 7 | 5 | NSD | NSD | -11 | -12 | -12 | NSD | NSD | -12 | -12 | -11 | NSD | -12 |
| STM1633 | 7 | 8 | NSD | NSD | -11 | -11 | -11 | NSD | NSD | -11 | -9 | -9 | NSD | -11 |
| STM1796 | 8 | 4 | NSD | NSD | -11 | -11 | -9 | NSD | NSD | -11 | -11 | -11 | -4 | -11 |
| STM2473 | 9 | 8 | -6 | NSD | -11 | -9 | -6 | -10 | -9 | NSD | -11 | -11 | NSD | -6 |
| STM2474 | 7 | 3 | NSD | NSD | -9 | -9 | NSD | -9 | -9 | NSD | -10 | -10 | -10 | NSD |
| STM3165 | 10 | 3 | -10 | -10 | -10 | -9 | -10 | -10 | -10 | NSD | -11 | -6 | NSD | -11 |
| STM3595 | 7 | 10 | NSD | NSD | -11 | -11 | -6 | NSD | NSD | -13 | -13 | -3 | NSD | -13 |
| STM4519 | 9 | 7 | NSD | NSD | -11 | -6 | -11 | -4 | -5 | NSD | -11 | -11 | -11 | -11 |

**COLOR KEY**
Not significantly different compared to WT
Down-regulated relative to WT (number represents fdd change)

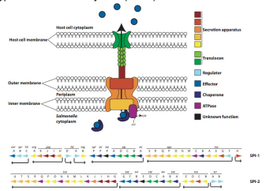
**Frequency (n = 12) -- number of regulatory mutants protein is coordinately down-regulated in Peptides -- number of peptides used to identify and quantify proteins coordinately regulated**

**Criteria for inclusion in candidate list of highly regulated proteins:**

- Protein must exhibit ≥ 2-fold reduction in expression in mutant compared to WT (ANOVA; p<0.05)
- Protein must be coordinately regulated in minimum of 7/12 regulator mutants
- Protein must be identified by ≥ 3 peptides

**Putative targets co-regulated with SPI-2 TTSS under MgM dilution growth condition**

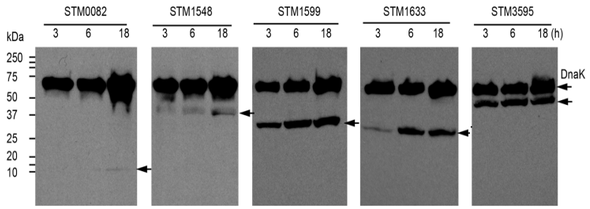
*Type three secretion system (TTSS)*




| Protein | Description | Fold Change | |
|---|---|---|---|
| STM1391 | Secretion system regulator (ssrB) | 3 | SPI-2 TTSS (Avg. fold change = 18) |
| STM1392 | Secretion system regulator (ssrA) | 12 | |
| STM1394 | Secretion system apparatus (ssaC) | 30 | |
| STM1396 | Secretion system effector (sseE) | 29 | |
| STM1397 | Secretion system effector (sseA) | 12 | |
| STM1399 | Secretion system chaperone (sscA) | 20 | |
| STM1402 | Secretion system effector (sseE) | 3 | |
| STM1407 | Secretion system apparatus (ssaH) | 27 | |
| STM1409 | Secretion system apparatus (ssaJ) | 25 | |
| STM1412 | Secretion system apparatus (ssaL) | 25 | |
| STM0082 | putative secreted protein | 90 | Proteins co-regulated with SPI-2 |
| STM1548 | putative S-adenosylmethionine:tRNA-rib | 48 | |
| STM1599 | Periplasmic dipeptidase for D-ala-D-ala | 34 | |
| STM1633 | putative periplasmic binding protein | 46 | |
| STM3595 | putative phosphatase | 307 | |

- SPI-2 TTSS - well characterized system critical for virulence and intra-macrophage replication and survival
- Proteins co-regulated with known virulence proteins are likely serve some type of support role in the bacterial processes related to virulence or may be important virulence factors themselves

### In vivo validation of LC-MS discovered biomarkers via Western blot




- Expression of STM0082, STM1548, STM1599, STM1633, STM3595 up-regulated upon infection of host macrophage cells, validating LC-MS results
- Results suggest STM0082, STM1548, STM1599, STM1633, STM3595 play a role in *Salmonella* infection of host cells

## Conclusions

- Label-free proteomics has helped identify and validate a set of proteins including STM0082, STM1548, STM1599, STM1633, and STM3595 that are:
  - Coordinately regulated by *Salmonella* regulatory proteins required for virulence
  - Co-regulated with the SPI-2 TTSS, a well characterized system critical for virulence and intra-macrophage replication and survival
- Confirmatory western blot analysis of the LC-MS discovered biomarker proteins following infection of host macrophage cells showed their expression was up-regulated validating the LC-MS results
- Collectively, the data suggest STM0082, STM1548, STM1599, STM1633, STM3595 play a role in *Salmonella* infection of host cells and may represent promising targets for therapeutic intervention

***Salmonella* proteomic data resource: http://www.proteomicsresource.org/**

## Acknowledgements

This work was funded by the National Institute of Allergy and Infectious Diseases NIH/DHHS through interagency agreement Y1-AI-4894-01. Samples were analyzed using instrumentation developed under the support of the NIH National Center for Research Resources (RR18522) and the U.S. Department of Energy Biological and Environmental Research (DOE/BER).

Significant portions of the work were performed in the Environmental Molecular Science Laboratory, a DOE/BER national scientific user facility at Pacific Northwest National Laboratory (PNNL) in Richland, Washington. PNNL is operated for the DOE by Battelle under contract DE-AC05-76RLO-1830.

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