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Use of high-throughput mass spectrometry to elucidate host–pathogen interactions in *Salmonella*

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Abstract

Capabilities in mass spectrometry are evolving rapidly, with recent improvements in sensitivity, data analysis and, most important from the standpoint of this review, much higher throughput, allowing analysis of many samples in a single day. This short review describes how these improvements in mass spectrometry can be used to dissect host–pathogen interactions using *Salmonella* as a model system. This approach has enabled direct identification of the majority of annotated *Salmonella* proteins, quantitation of expression changes under various *in vitro* growth conditions and new insights into virulence and expression of *Salmonella* proteins within host cells. One of the most significant findings is that a relatively high percentage of all the annotated genes (>20%) in *Salmonella* are regulated post-transcriptionally. In addition, new and unexpected interactions have been identified for several *Salmonella* virulence regulators that involve protein-protein interactions, suggesting additional functions of these regulators in coordinating virulence expression. Overall

high-throughput mass spectrometry provides a new view of host–pathogen interactions, emphasizing the protein products and defining how protein interactions determine the outcome of infection.

Keywords

host–pathogen interaction; intracellular pathogen; mass spectrometry; proteomics; *Salmonella*; virulence

The model system

Salmonella is a common and important pathogen, which infects 200 million–1.3 billion people worldwide, with over 3 million deaths annually [1]. *Salmonella* is a model without equal for studying intracellular pathogenesis because of its established genetics and the availability of a simple and inexpensive animal model – the mouse. Because *Salmonella* remain within professional phagocytic cells throughout the course of infection in the mouse, the ability of *Salmonella* to successfully infect and replicate within monocytes is frequently used as an *in vitro* surrogate for successful infection of the host [2,3]. Elucidating both the general strategies and precise mechanisms of *Salmonella* pathogenesis will provide an exemplar for understanding other bacterial pathogens.

Salmonella is a Gram-negative facultative bacterial pathogen that can infect diverse hosts including birds, reptiles and mammals. *Salmonella enterica* serovar Typhimurium (*S. Typhimurium* or STM) causes a self-limiting gastroenteritis in humans, whereas the closely related *S. enterica* serovar Typhi (*S. Typhi*) causes frequently fatal typhoid fever. With the continuing emergence of multidrug-resistant strains such as phage type DT104 [4] and nearly monthly food contamination outbreaks occurring in the USA, as reported by the CDC [101], the public health threat is significant, *S. Typhi* is exquisitely host adapted to humans and some higher primates and has no experimental animal model. *S. Typhimurium* causes typhoid fever in man and a similar disease in mice, and is consequently the primarily studied *Salmonella* pathogen for scientific research. The LD₅₀, duration of the infection, titers in infected organs and the general outcome of the disease depend on three things: the route of inoculation, the strain of *S. Typhimurium* used to infect and the genetic background of the mouse infected. The two most commonly used mouse models are C57BL/6 and Balb/c. Both of these strains are susceptible to *S. Typhimurium* infection and die following either intragastric or intraperitoneal infection with the strain used in this work (14028). The absence or functional disruption of the host protein known as natural resistance-associated macrophage protein-1 (Nrampl; Slc11a1) plays a crucial role in determining the susceptibility of these two strains of mice to *Salmonella* infection [5–8]. By contrast, SvJ129x mice have a functional Nrampl and are resistant to a fatal infection following high-dose oral inoculation, but instead develop a chronic infection [9]. Using a liquid chromatography coupled to mass spectrometry (LC-MS)-based proteomics approach, as described in more detail below, we have identified a *Salmonella* operon that is expressed in response to Nrampl and is essential for *Salmonella* virulence [10]. There are many other strains of mice that contain mutations in specific antimicrobial components normally expressed by professional phagocytic cells [11]. These mouse derivatives will be invaluable in analyzing host–pathogen interactions and in constructing a computational model of *Salmonella*-host interaction.

In atypical infection, *Salmonella* enters the body upon the ingestion of contaminated food, passes through the stomach to the small intestine and becomes systemic in one of two ways; either by invading M cells to reach the lymphatic system or via carriage within CD18⁺ monocytes directly to the bloodstream [12–14]. Remarkably, *Salmonella* can become systemic within minutes of oral infection in man or mice by hijacking phagocytic cells [15]. This rapid

dissemination is mediated by the specific secretion of *Salmonella* proteins that bind to parts of the host cell motility apparatus [16]. Once *Salmonella* reaches its preferred site of replication, the spleen and liver, it initially replicates within neutrophils [14,16–19] and eventually macrophages/monocytes, and can be found in virtually every white blood cell [14]. Furthermore, the host is made more hospitable to the invading *Salmonella* through diverse virulence factors, which are encoded within horizontally transferred DNA sequences (pathogenicity islands), then expressed and secreted in a highly regulated process at different stages of host infection (Figure 1) [16,17,20–24].

Adaptation to the host & disruption of host biology is mediated through proteins

The ability of *Salmonella* to infect its host, survive in the intracellular environment and replicate requires the transport of virulence factors and other effector proteins to the host cell's cytoplasm [25,26] through a type III secretion system [27–31]. Two type III secretion systems are encoded at different sites on the chromosome of all *Salmonella enterica*. The secretion machinery encoded by *Salmonella* pathogenicity island one (*SPI-1*) provides *S. enterica* with the ability to invade epithelial cells and invoke the inflammatory response [27–30], while the secretory system encoded by *SPI-2* facilitates the secretion of proteins that allow the bacteria to thrive within epithelial cells and even professional phagocytes [27,31–36]. *Salmonella* replicates within phagocytic cells by preventing maturation of the phagocytic vesicle, thereby blocking exposure to many lysosomal proteins [37–39], and then kills the host cell, allowing the bacteria to spread to neighboring cells, presumably within apoptotic bodies [40–42].

The focus of this review is the *Salmonella* proteome as investigated via mass spectrometry as an approach to understanding pathogenesis. In this context, it is important to point out that while the central dogma asserts that information flows from DNA to RNA to proteins, this does not mean that regulation only occurs at the level of the gene. Regulation of proteins can occur transcriptionally, translationally and post-translationally through modifications, turnover and environment-mediated changes in activity. Transcriptional regulation is itself complex and not the focus of this review. Translational control has been of increasing interest in many biological systems, especially when mediated through small RNAs (sRNAs). Recent studies have implicated three translational regulators - *csrA*, small protein B (*smpB*) and host factor for QB phage replication (*Hfq*) - as major virulence factors. Regulation of the *SPI-1* by *csrA* is well documented [43–45], and a mutation in *smpB* was one of the first macrophage-sensitive mutants identified [2,43,46]. We have found that *smpB* and *Hfq* are potent general translational regulators of virulence [ANSONG ET AL., & YOON ET AL., UNPUBLISHED DATA]; virulence studies of *Hfq* mutants in *S. Typhimurium* have been recently published [44]. The observation that these three virulence factors operate at the level of translating RNA into protein highlights the need to directly measure proteins, and not just transcripts, in order to develop a global understanding of *Salmonella* pathogenesis.

High-throughput mass spectrometry-based proteomics

The introduction of new MS-based techniques over the past decade has opened new approaches to measuring and understanding the dynamic protein complement (i.e., the proteome) of host-pathogen systems [45]. There are two basic biological questions that proteomics can address. The first is that often gene transcript abundances do not reflect protein abundances and, of course, a global analysis of abundance for each protein is a better surrogate measurement of protein functions. Second, each protein may be modified by cleavage, lipidation, phosphorylation, glycosylation and so on to attain full function; therefore MS analysis may provide useful clues regarding the functional state of a biological system. *Salmonella* must rapidly adapt to multiple hostile environments in order to successfully replicate in its host, and

it is therefore reasonable to assume that regulation at all levels will be used to rapidly and specifically tailor responses to changing environments. The presence of an actively transcribed mRNA molecule will clearly reduce the time it takes to express a virulence factor (protein), although it will cost the bacteria energy for transcription and subsequent turnover of the nucleotides. Certainly proteins and their modifications are best studied using MS-based proteomic technologies, and this brief review will provide an introduction to studying host-pathogen interactions in more detail.

Among the many new MS-based approaches to studying proteomes, the arguably most effective has been the variations on the 'bottom-up' approach, typified by Yates and coworkers [45,47,48]. In this more traditional approach, proteins are isolated from the biological sample of interest; this sample could be a tissue, host cells, pathogens or even subcellular fractions (e.g., cytoplasm, membranes and organelles). The isolated proteins are then denatured and digested with a site-specific protease, which is analogous to restriction enzymes for DNA. The most widely used protease is trypsin, which cleaves proteins into smaller pieces (peptides) on the C terminal side of arginine and lysine residues leaving fragments with a median size generally smaller than 30 amino acid residues. The resulting peptides are highly amenable to separation via reversed-phase liquid chromatography and this is generally used in conjunction with MS to allow much greater depth (dynamic range) of proteome coverage.

Tandem mass spectrometry (MS/MS) is used to select and fragment a subset of peptides, allowing protein identification from the genomic sequence of the organism. A number of informative studies have used this approach to characterize *Salmonella*, including studies of protein abundance changes related to antibiotic resistance [49–51] and an extensive study of various sample preparation methods [52].

The accurate mass and time (AMT) tag approach was developed to circumvent the 'undersampling' associated with LC-MS/MS bottom-up approaches, providing improved throughput and sensitivity [53]. The experimental isolation and digestion of proteins is performed in the same fashion as above, but instead of using MS/MS, which requires isolation and fragmentation of each peptide, high resolution nanocapillary LC [54] is combined with very high accuracy mass measurements, that is, peptide AMT tags. However, extensive LC-MS/MS measurements are used to establishing the AMT tag database of confident tryptic peptides, enabling subsequent analysis of samples with much higher throughput using the LC elution times and AMT tags of peptides previously identified. Proteins are generally identified with high confidence from multiple AMT tags, along with their relative abundances. The AMT tag proteomics approach is being applied to understanding host-*Salmonella* interactions, and is particularly useful in experiments with limited sample quantities or those for which a large number of samples must be compared.

Despite many successful physiological, biochemical and genetic methods used to determine the key virulence determinants encoded by this organism, the sheer number of uncharacterized reading frames observed within the *S. enterica* genome suggests that many more virulence factors remain to be discovered. As an example, using the traditional proteomics approach described above 10 compare *Salmonella* proteins expressed under typical laboratory conditions and conditions that mimic the intracellular environment resulted in the identification of a diverse spectrum of proteins under the distinct growth conditions (as well as to establish an initial AMT tag database) [55]. Many of these were associated with known metabolic requirements in the nutrient-poor environment present within the *Salmonella* containing vacuole (SCV). In fact, more than 3400 proteins of a total of 4457 annotated open reading frames were identified and compared across four growth conditions.

Analysis of *Salmonella* protein expression under diverse growth conditions

When grown to late logarithmic phase in rich, relatively high-salt medium (100 mM NaCl), *S. Typhimurium* is known to express many genes that are required for invasion of epithelial cells. By contrast, bacteria grown in an acidic, iron, magnesium and phosphate-depleted minimal medium (MgM) upregulate expression of many genes required for systemic infection such as those encoded within SPI-2 [56]. Protein abundance comparisons from bacteria grown under each of these conditions indicate that the majority of proteins do not change significantly. However, the expression of subsets of proteins was largely restricted to one of three culture conditions. For example, cells grown in MgM had a higher abundance of Mg²⁺ transport proteins than found in other growth conditions. A more virulent *S. Typhimurium* strain (strain 14028; LD₅₀ ~10⁶ intragastric, LD₅₀ <10¹ intraperitoneal) was also cultured under these same growth conditions and the results were directly compared with those obtained for the laboratory strain LT2. Among a number of proteins displaying a higher abundance in strain 14028 were the products of the *pdu* operon, which encodes enzymes required for propanediol utilization [55,57]. In another study, Coldham and Woodward found additional proteins present that appeared to be specifically regulated by anaerobic growth [58].

Expression within host cells

Certain inbred strains of mice, such as Balb/c and C57/B16, are more sensitive to intracellular pathogens because they lack the gene that encodes a natural resistance protein (Nramp1 or Slc11a1). The difference in susceptibility is due to the expression of a vacuolar ion channel that removes Fe²⁺ and Mn²⁺ ions from within the phagocytic vacuole of macrophages and other professional phagocytic cells. To cause infection, *Salmonella* must alter its expression profile following phagocytosis and respond to the presence of specific innate immune mechanisms such as Nramp1. To identify new colonization and virulence factors that mediate *S. Typhimurium* pathogenesis, we isolated bacterial cells from RAW 264.7 (Nramp^{+/-}) macrophages at various time points following infection [10]. The AMT tag proteomics approach was used to detect changes in *Salmonella* protein abundance due to the relatively small sample quantities available. While this presented significant challenges owing to the high abundances of contaminating host proteins, a total of 315 *S. Typhimurium* proteins were identified from isolated bacterial cells, most of which were housekeeping proteins. However, 39 bacterial proteins were strongly induced after infection, suggesting their involvement in modulating colonization and infection. Of the 39 induced proteins, six are specifically modulated by Nramp1 activity, including STM3117–3119, whose time-dependent abundance changes were confirmed using western blot analysis. These genes appear to be located in an operon in which the first gene is a *lysR* type regulator (STM3120). Deletion of any one of these genes resulted in a dramatic reduction of virulence in mice [Yoon & Heffron, Unpublished data]. In addition, STM3117 deletion mutant was unable to survive in RAW 264.7 macrophages, demonstrating the critical involvement of STM3117 in promoting the replication of *S. Typhimurium* inside macrophages. A predicted function for STM3117–3119 is modification of the peptidoglycan layer of the cell wall, although this has not been established conclusively [10].

Comparison of expression in related bacteria: *Salmonella Typhimurium* & Typhi proteomes

Typhoid fever is a potentially fatal disease caused by the bacterial pathogen *S. Typhi*. *S. Typhi* infection is a complex process that involves numerous bacterially encoded virulence determinants, and these are thought to confer both stringent human host-specificity and a high mortality rate. In a recent study, we used the bottom-up proteomics strategy to investigate the proteome of logarithmic, stationary phase and low pH/low magnesium (MgM) *S. Typhi*

cultures. This report represented the first large-scale comprehensive characterization of the *S. Typhi* proteome [59]. Our analysis identified a total of 2066 *S. Typhi* proteins. In an effort to identify putative *S. Typhi*-specific virulence factors, we then compared our *S. Typhi* results to those obtained in a previously published study of the *S. Typhimurium* proteome under similar conditions [55]. Comparative proteomic analysis of *S. Typhi* strain Ty2 and *S. Typhimurium* strain LT2 revealed a subset of highly expressed proteins unique to *S. Typhi* that were exclusively detected under conditions that are thought to mimic the infective state in macrophage cells. These proteins included CdtB, HlyE and gene products of t0142 t1108, t1109, t1476 and t1602. The differential expression of T1108, T1476 and HlyE was confirmed by western blot analysis. Our observations, together with the current literature, suggest that this subset of proteins may play a role in *S. Typhi* pathogenesis and human—host specificity.

Mass spectrometric dissection of protein-protein interactions

An exciting new area of research is the application of MS to the identification of transient protein interactions within living cells. These are interesting and elusive events because they take place during signal transduction and are critical for cellular response. One way to identify the interacting partners is by crosslinking them chemically within a live cell. Crosslinkers can covalently bond two interacting proteins in a complex system and, depending on the chemistry, this can range from nonspecific to highly specific. The advantage of crosslinking before cell lysis is that it can fix strong, weak and even transient interactions *in vivo* [60–62]. New interest in this area is prompted by a convergence of recent advances in high-throughput mass spectrometry, the availability of highly specific crosslinking reagents and new tandem affinity tags suitable to purify protein complexes before MS analysis. In addition to being suitable for analyzing protein interactions within the cell, the crosslinking reagent should be membrane permeable. The most common crosslinking reagent of this nature is formaldehyde as it is completely membrane permeable and highly reactive with both proteins and nucleic acids [62]. Formaldehyde forms unstable methylol adducts and Schiff's bases in the amino acid side chain of the proteins (generally, lysine, arginine, tyrosine and tryptophan residues). This produces a wide range of crosslinking in the protein complexes *in vivo*, although lysine and tryptophan are the preferred targets when crosslinking proteins for short times at 25°C [62]. To form stable protein complexes via Schiff's bases, the separation must be less than 2.7 Å, and therefore formaldehyde reacts only with proteins in immediate proximity. An additional advantage is that formaldehyde crosslinks can be reversed by hydrolysis at a reasonable temperature (90–95°C). Most research has focused on attachment of an affinity tag that can be used to purify crosslinked proteins, which are then identified by MS. As one example of examining specific interactions, Huang, Kaiser and coworkers have developed an affinity tag that allows purification under denaturing conditions and used it to identify highly unstable ubiquitinated proteins in *Saccharomyces cerevisiae* [60,61]. We have modified their approach for application in bacteria specifically to a variety of regulatory proteins that are essential for *Salmonella* virulence. Of the four regulators examined, we found that as expected HimA and HimD could be purified as a complex. Furthermore, we were able to identify PhoQ's interaction with PhoP, although this interaction must be transient. The analysis also revealed several unexpected interactions of the two response regulators OmpR and PhoP, including direct interaction with Hfq, perhaps explaining PhoP translational regulation of SsrA [63]. There is no doubt that a combination of heavy atom derivatives of formaldehyde, more sophisticated computer algorithms [64] and new MS methods will be used to dissect host–pathogen interactions in the next few years.

Recently, stable isotope labeling with amino acids in cell culture (SILAC) has been used to help determine host targets of one specific secreted effector, SopB [65]. This method uses isotopic labeling to help discern specific from nonspecific background interactions. For one part of the experiment no special isotopic distribution of the peptides were used (i.e., cells that

contain a secreted effector with an enrichable tag) and for the other part of the experiment (i.e., cells grown without the enrichable secreted effector) specific amino acids are labeled with heavy isotopes of hydrogen, nitrogen and/or carbon. The cells are then mixed in equal quantities and the tagged SopB is immunoprecipitated. A bottom-up proteomics experiment is performed using an instrument capable of high resolution mass measurement to allow the different isotopes to be discerned and the ratios compared. This approach allows for the presence of a protein in the negative control that would typically be removed by the experiment described above.

Analysis of the native peptidome of *Salmonella* Typhimurium

Peptides are typically defined as polyamino acid chains of less than 100 amino acid residues, and in the context of proteomics they are usually the result of a protease added to a protein sample. Analysis of peptides that are the result of either small protein-coding regions or native proteolysis is of great interest, because these native peptides could have unusual and powerful biological properties. Native peptides are less likely to have enzymatic activity because they are too small to encode a complex active site, but cytokines and neuropeptides are examples of peptides with potent biological function. Typically, the identification of a native peptide by the AMT tag or other MS approach is difficult simply because trypsin protease may only cleave a few peptides. Identification by SEQUEST or other computer algorithms generally requires two or more peptides be identified before they can be assigned to a specific protein with confidence. We have used comparative native peptidomics, defined as analysis of this native low-molecular-weight polypeptide fraction, to study STM cultured under a variety of growth conditions, including two minimal nutrient media designed to roughly mimic the macrophage phagosomal environment. Native peptides from cleared cell lysates were enriched using isopropanol extraction and analyzed using both LC-MS/MS and LC-Fourier transform ion cyclotron resonance (FTICR) MS. More than 5000 peptides were identified, originating from 682 proteins [57,102,103]. Analysis clearly indicates that, compared with *Salmonella* cultured in the rich medium, cells cultured in a phagosome-mimicking medium had dramatically higher abundances of a wide variety of degraded proteins, especially from ribosomal proteins. *Salmonella* from the same cultures were also analyzed using traditional bottom-up proteomic methods, and when the peptidomic and proteomic data were analyzed together, two clusters of proteins targeted for proteolysis were identified. Many of these peptides undoubtedly arose because the stress conditions and minimal media reduce growth rate and trigger degradation of ribosomal proteins to recycle amino acids. Paradoxically, stress proteins and biosynthetic operons that are highly induced under these growth conditions give rise to a second abundant class of peptides. These are most likely a consequence of starvation reducing the fidelity of translation that, in turn, results in high turnover of the mistranslated protein [66]. In addition, nine proteins were identified that had not been annotated, seven of which are co-encoded within another annotated gene. It is also very likely that some of the identified peptides are biologically active although their precise function remains to be clarified [67,68]. Comparison of bacterial genomes demonstrates a predilection for antigens that are related to those of their host, presumably to avoid immunologic surveillance [69] and such antigens were detected in this study. The biological significance of this molecular mimicry is that it can result in crossreactivity between the bacterial epitope and its human counterpart, breaking immunologic tolerance resulting in Reiter's syndrome, Guillain-Barre syndrome and other sequelae that often follow bacterial infections.

Comparison between transcriptional profiles & the proteome using high-throughput mass spectrometry

The global level of translational regulation in bacteria is not known, nor is the role that this may play in *Salmonella* pathogenesis. MS is the ideal tool to differentiate between

transcriptional and translational regulation and to determine whether *Salmonella* uses post-transcriptional regulation during infection. Mutation of any of the known transcriptional regulators, Hfq, SmpB or CsrA, results in a profound loss of virulence. Samples of *Salmonella* were prepared from both parental and isogenic derivatives of Hfq and *smpB* that were grown under widely different conditions and divided for analysis by both transcriptional profiling and proteomics. The results were completely unexpected - Hfq regulates expression of at least 20% of the entire proteome of *Salmonella*, while even *smpB*, thought to be limited to trans-translation, regulated at least 4% of all proteins. Hfq modulates a broad range of cellular processes, including central metabolism, fatty acid metabolism, quorum sensing, LPS biosynthesis, two-component regulators, stress-response genes and virulence factors. This was the first work to show that *smpB* might be a general translational regulator and that Hfq has a profound effect on translation throughout the entire genome [ANSONG ET AL., UNPUBLISHED DATA].

Future perspective

The central question in pathogenesis is how a pathogen interacts with its host. On the bacterial side is a plethora of secreted proteins; on the host side are the formidable barriers of the innate and adaptive immune system. The Gram-negative intracellular pathogens alone encode hundreds of effector proteins that are injected into host cells during infection. The function of most of these proteins remains a mystery. In *Salmonella* alone, there are over 35 identified effectors and there are very likely to be more, given that 1700 open reading frames are annotated as putative, unknown or conserved unknown. Of these 35 effectors, a cellular target is known for only a few [16,70–72]. The MS-based methods will continue to have a huge impact on these studies in several ways. The objective of these experiments was to identify the interacting proteins and the consequence of their interactions. Improvements in MS sensitivity, crosslinking reagents, computer algorithms to identify crosslinked peptides and tandem affinity probes will allow identification of even transiently interacting cellular proteins. Lipid and metabolic changes that take place in response to the host are also amenable to LC-MS analysis. LC-MS approaches allow rapid identification of bio-molecules that can provide an integrated map of host–pathogen interactions. Most of the studies have either been *in vitro*, using conditions that mimic the host, or in cell culture, but by labeling the bacteria with a fluorescent protein, it has recently been possible to purify bacteria from an infected host and identify some of the proteins expressed *in vivo* via MS [73], perhaps opening the door to a direct systematic approach to studying host–pathogen interactions even within the host.

Future improvements on the horizon for LC-MS-based methods of studying host–pathogen interactions include increased throughput methods that may reduce or even eliminate the need for LC by using gas-phase separation methods. This promises to reduce analysis time for an entire proteome from hours to minutes. Novel ‘lab-on-a-chip’ design may allow for analysis of very small samples that may even include single-cell analyses or very precise subcellular fractionation methods that could improve the ability to measure host–pathogen mixed samples. Further development of improved crosslinking reagents and interpretation software will allow researchers to discover the sites of interaction between proteins in a global fashion, leading to a convergence with structural biology.

Executive summary

Introduction

- New capabilities in mass spectrometry (MS) were used to study *Salmonella* host–pathogen interactions.

- Use of different *in vitro* growth conditions enabled identification of most known (annotated) *Salmonella* proteins, and changes in protein abundance as a function of growth conditions were quantified.
- More than 20% of annotated *Salmonella* genes are regulated post-transcriptionally.
- Novel protein-protein interactions identified for *Salmonella* virulence regulators.

***Salmonella* model system**

- *Salmonella* is a highly prevalent Gram-negative bacterial pathogen with significant morbidity and mortality worldwide.
- Murine infection with *Salmonella* Typhimurium provides a useful model system for human infection with *Salmonella* Typhi.

Adaptation to host

- Secretion of protein virulence factors through a type III secretion system is vital to *Salmonella* infection, intracellular survival and replication.
- Three translational regulators in *Salmonella* - csrA, smpB and Hfq - are potent regulators of virulence.

Mass spectrometry-based proteomics

- MS allows direct measurement of protein abundance and post-translational modifications.
- Accurate mass and time (AMT) tag proteomics provides several advantages for high-throughput proteomic studies, especially improved sensitivity and throughput.

***Salmonella* protein expression**

- Comparison of protein expression under four different growth conditions and within host cells provided insight into proteins required for intracellular survival and virulence.
- Comparison of *S. Typhimurium* and *S. Typhi* identified Typhi-specific proteins implicated in virulence and human host specificity.
- Use of formaldehyde crosslinking allowed identification of transient protein-protein interactions
- Analysis of a native *Salmonella* ‘peptidome’ in different growth conditions demonstrated an additional complexity in understanding the proteome.

Conclusion & future perspective

- Parallel analyses of transcripts and proteins are essential for a comprehensive understanding of *Salmonella* pathogenesis.
- Gas phase separations and ‘lab-on-a-chip’ designs will further improve throughput and sensitivity; combined with improved crosslinking reagents, in depth analyses of protein-protein interactions will soon be feasible.

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Bibliography

Paper of special note have been highlighted as:

▪ of interest

▪▪ of considerable interest

1. Coburn B, Grassl GA, Finlay BB. *Salmonella*, the host and disease: a brief review. *Immunol. Cell. Biol* 2007;85:112–118.118 [PubMed: 17146467] ▪▪ Presents an overview of *Salmonella*–host interaction.
2. Fields PI, Swanson RV, Haidaris CG, Heffron F. Mutants of *Salmonella* Typhimurium that cannot survive within the macrophage are avirulent. *Proc. Natl Acad. Sci. USA* 1986;83:5189–5193.5193 [PubMed: 3523484] ▪ Convincingly demonstrates the key role of macrophages in systemic infections.
3. Finlay BB, Brumell JH. *Salmonella* interactions with host cells: *in vitro* to *in vivo*. *Philos. Trans. R. Soc. Lond. B. Biol. Sci* 2000;355:623–631. [PubMed: 10874735]
4. Glynn MK, Bopp C, Dewitt W, Dabney P, Mokhtar M, Angulo FJ. Emergence of multidrug-resistant *Salmonella enterica* serotype Typhimurium DT104 infections in the United States. *N. Engl. J. Med* 1998;338:1333–1338. [PubMed: 9571252]
5. O'Brien AD, Rosenstreich DL, Taylor BA. Control of natural resistance to *Salmonella* Typhimurium and *Leishmania denovani* in mice by closely linked but distinct genetic loci. *Nature* 1980;287:440–442. [PubMed: 7001248]
6. Swanson RN, O'Brien AD. Genetic control of the innate resistance of mice to *Salmonella* Typhimurium: *Ity* gene is expressed *in vivo* by 24 hours after infection. *J. Immunol* 1983;131:3014–3020. [PubMed: 6315821]
7. Sebastiani G, Olien L, Gauthier S, et al. Mapping of genetic modulators of natural resistance to infection with *Salmonella* Typhimurium in wild-derived mice. *Genomics* 1998;47:180–186. [PubMed: 9479490]
8. Govoni G, Canonne-Hergaux F, Pfeifer CG, et al. Functional expression of *Nrampl* *in vitro* in the murine macrophage line RAW264.7. *Infect. Immun* 1999;67:2225–2232. [PubMed: 10225878]
9. Lawley TD, Chan K, Thompson LJ, Kim CC, Govoni GR, Monack DM. Genome-wide screen for *Salmonella* genes required for long-term systemic infection of the mouse. *PLoS Pathog.* 2, ell. 2006 ▪ Uses state-of-the-art methods to identify all the *Salmonella* genes necessary to cause a persistent mouse infection.
10. Shi L, Adkins JN, Coleman JR, et al. Proteomic analysis of *Salmonella enterica* serovar Typhimurium isolated from RAW 264.7 macrophages: identification of a novel protein that contributes to the replication of serovar Typhimurium inside macrophages. *J. Biol. Chem* 2006;281:29131–29140.29140 [PubMed: 16893888] ▪▪ Proteomics of *Salmonella* grown in host macrophages.
11. Rutschmann S, Hoebe K. Dissecting innate immunity by germline mutagenesis. *Immunology* 2008;123:459–468. [PubMed: 18205789]
12. Baumler AJ, Tsolis RM, Valentine PJ, Ficht TA, Heffron F. Synergistic effect of mutations in *invA* and *lpfC* on the ability of *Salmonella* Typhimurium to cause murine typhoid. *Infect. Immun* 1997;65:2254–2259. [PubMed: 9169760]
13. Vazquez-Torres A, Jones-Carson J, Baumler AJ, et al. Extraintestinal dissemination of *Salmonella* by CD18-expressing phagocytes. *Nature* 1999;401:804–808. [PubMed: 10548107]

14. Worley MJ, Nieman GS, Geddes K, Heffron F. *Salmonella* Typhimurium disseminates within its host by manipulating the motility of infected cells. *Proc. Natl Acad. Sci. USA* 2006;103:17915–17920. [PubMed: 17095609] ▪ First paper to demonstrate that a bacterial pathogen controls its own course of infection.
15. Gerichter CB. The dissemination of *Salmonella* Typhi, *S. paratyphi* A and *S. paratyphi* B through the organs of the white mouse by oral infection. *J. Hyg* 1960;58:307–319. [PubMed: 13704606]
16. Geddes K, Cruz F, Heffron F. Analysis of cells targeted by *Salmonella* type III secretion *in vivo*. *PLoS Pathog* 2007;3:e196. [PubMed: 18159943] ▪ Describes the first detailed study of the types of infected cells and the cells targeted by type III secretion *in vivo* in a mouse infection model.
17. Raz E, Zlokarnik G, Tsien RY, Driever W. β -lactamase as a marker for gene expression in live zebrafish embryos. *Dev. Biol* 1998;203:290–294. [PubMed: 9808780]
18. Dunlap NE, Benjamin WU Jr, Berry AK, Eldridge JH, Briles DE. A ‘safe-sire’ for *Salmonella* Typhimurium is within splenic polymorphonuclear cells. *Microb. Pathog* 1992;13:181–190. [PubMed: 1291841]
19. Vazquez-Torres A, Fang FC. Cellular routes of invasion by enteropathogens. *Curr. opin. Microbiol* 2000;3:54–59. [PubMed: 10679413]
20. Zhang J, Campbell RE, Ting AY, Tsien RY. Creating new fluorescent probes for cell biology. *Nat. Rev. Mol. Cell Biol* 2002;3:906–918. [PubMed: 12461557]
21. Lawrence JG, Ochman H, Hard DL. Molecular and evolutionary relationships among enteric bacteria. *J. Gen. Microbiol* 1991;137:1911–1921. [PubMed: 1955870]
22. Ochman H, Bergthorsson U. Genome evolution in enteric bacteria. *Curr. Opin. Genet. Dev* 1995;5:734–738. [PubMed: 8745071]
23. Ochman H, Groisman EA. Distribution of pathogenicity islands in *Salmonella* spp. *Infect. Immun* 1996;64:5410–5412. [PubMed: 8945597]
24. Patel JC, Rossanese OW, Galan JE. The functional interface between *Salmonella* and its host cell: opportunities for therapeutic intervention. *Trends Pharmacol. Sci* 2005;26:564–570. [PubMed: 16182381] ▪ Overview of how *Salmonella* alters host cell functions, highlighting much of the work on cell entry from the Galan laboratory; emphasizes how the interactions may provide new therapeutic targets.
25. Michiels T, Cornelis GR. Secretion of hybrid proteins by the *Yersinia* Yop export system. *J. Bacteriol* 1991;173:1677–1685.
26. Rosqvist R, Forsberg A, Wolf-Watz H. Intracellular targeting of the *Yersinia* YopE cytotoxin in mammalian cells induces actin microfilament disruption. *Infect. Immun* 1991;59:4562–4569. [PubMed: 1937815]
27. Galan JE, Ginocchio C, Costeas P. Molecular and functional characterization of the *Salmonella* invasion gene *invA*: homology of InvA to members of a new protein family. *J. Bacteriol* 1992;174:4338–4349. [PubMed: 1624429]
28. Galyov EE, Wood MW, Rosqvist R, et al. A secreted effector protein of *Salmonella dublin* is translocated into eukaryotic cells and mediates inflammation and fluid secretion in infected ileal mucosa. *Mol. Microbiol* 1997;25:903–912. [PubMed: 9364916]
29. Lee CA, Silva M, Siber AM, Kelly AJ, Galyov E, McCormick BA. A secreted *Salmonella* protein induces a proinflammatory response in epithelial cells, which promotes neutrophil migration. *Proc. Natl Acad. Sci. USA* 2000;97:12283–12288. [PubMed: 11050248]
30. Hapfelmeier S, Ehrbar K, Stecher B, Barthel M, Kremer M, Hardt WD. Role of the *Salmonella* pathogenicity island 1 effector proteins SipA, SopB, SopE, and SopE2 in *Salmonella enterica* subspecies 1 serovar Typhimurium colitis in streptomycin-pretreated mice. *Infect. Immun* 2004;72:795–809. [PubMed: 14742523]
31. Groisman EA, Sturmoski MA, Solomon FR, Lin R, Ochman H. Molecular, functional, and evolutionary analysis of sequences specific to *Salmonella*. *Proc. Natl Acad. Sci. USA* 1993;90:1033–1037. [PubMed: 8430070]
32. Galan JE, Ginocchio C. The molecular generic bases of *Salmonella* entry into mammalian cells. *Biochem. Soc. Trans* 1994;22:301–306. [PubMed: 7958312]

33. Hensel M, Shea JE, Baumler AJ, Gleeson C, Blattner F, Holden DW. Analysis of the boundaries of *Salmonella* pathogenicity island 2 and the corresponding chromosomal region of *Escherichia coli* K-12. *J. Bacteriol* 1997;179:1105–1111. [PubMed: 9023191]
34. Baumler AJ, Tsolis RM, Ficht TA, Adams LG. Evolution of host adaptation in *Salmonella enterica*. *Infect. Immun* 1998;66:4579–4587. [PubMed: 9746553]
35. Shea JE, Beuzon CR, Gleeson C, Mundy R, Holden DW. Influence of the *Salmonella* Typhimurium pathogenicity island 2 type III secretion system on bacterial growth in the mouse. *Infect. Immun* 1999;67:213–219. [PubMed: 9864218]
36. Hensel M. *Salmonella* pathogenicity island 2. *Mol. Microbiol* 2000;36:1015–1023. [PubMed: 10844687]
37. Knodler LA, Vallance BA, Hensel M, Jackel D, Finlay BB, Steele-Mortimer O. *Salmonella* type III effectors PipB and PipB2 are targeted to detergent-resistant microdomains on internal host cell membranes. *Mol. Microbiol* 2003;49:685–704. [PubMed: 12864852]
38. Drecktrah D, Knodler LA, Steele-Mortimer O. Modulation and utilization of host cell phosphoinositides by *Salmonella* spp. *Infect. Immun* 2004;72:4331–4335. [PubMed: 15271887]
39. Birmingham CL, Brumell JH. Autophagy recognizes intracellular *Salmonella enterica* serovar Typhimurium in damaged vacuoles. *Autophagy* 2006;2:156–158. [PubMed: 16874057]
40. Chen LM, Kaniga K, Galan JE. *Salmonella* spp. are cytotoxic for cultured macrophages. *Mol. Microbiol* 1996;21:1101–1115. [PubMed: 8885278]
41. Chen Y, Smith MR, Thirumalai K, Zychlinsky A. A bacterial invasin induces macrophage apoptosis by binding directly to ICE. *EMBO J* 1996;15:3853–3860. [PubMed: 8670890]
42. Lindgren SW, Stojiljkovic I, Heffron F. Macrophage killing is an essential virulence mechanism of *Salmonella* Typhimurium. *Proc. Natl Acad. Sci. USA* 1996;96:4197–4201. [PubMed: 8633040]
43. Baumler AJ, Heffron F. Mosaic structure of the *smpB-nrdE* intergenic region of *Salmonella enterica*. *J. Bacteriol* 1998;180:2220–2223. [PubMed: 9555907]
44. Sittka A, Pfeiffer V, Tedin K, Vogel J. The RNA chaperone Hfq is essential for the virulence of *Salmonella* Typhimurium. *Mol. Microbiol* 2007;63:193–217. [PubMed: 17163975]
45. Aebersold R, Mann M. Mass spectrometry-based proteomics. *Nature* 2003;422:198–207. [PubMed: 12634793] • Excellent higher level review of proteomics methods.
46. Baumler AJ, Kusters JG, Stojiljkovic I, Heffron F. *Salmonella* Typhimurium loci involved in survival within macrophages. *Infect. Immun* 1994;62:1623–1630. [PubMed: 8168923]
47. Washburn MP, Wolters D, Yates JR 3rd. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat. Biotechnol* 2001;19:242–247. [PubMed: 11231557]
48. Wolters DA, Washburn MP, Yates JR 3rd. An automated multidimensional protein identification technology for shotgun proteomics. *Anal. Chem* 2001;73:5683–5690. [PubMed: 11774908]
49. Coldham NG, Randall LP, Piddock LJ, Woodward MJ. Effect of fluoroquinolone exposure on the proteome of *Salmonella enterica* serovar Typhimurium. *J. Antimicrob. Chemother* 2006;58:1145–1153. [PubMed: 17062612]
50. Karatzas KA, Randall LP, Webber M, et al. Phenotypic and proteomic characterization of multiply antibiotic-resistant variants of *Salmonella enterica* serovar Typhimurium selected following exposure to disinfectants. *Appl. Environ. Microbiol* 2008;74:1508–1516. [PubMed: 18083849]
51. Webber MA, Coldham NG, Woodward MJ, Piddock LJ. Proteomic analysis of triclosan resistance in *Salmonella enterica* serovar Typhimurium. *J. Antimicrob. Chemother* 2008;62:92–97. [PubMed: 18388111]
52. Nunn BL, Shaffer SA, Scherl A, et al. Comparison of a *Salmonella* Typhimurium proteome defined by shotgun proteomics directly on an LTQ-FT and by proteome pre-fractionation on an LCQ-DUO. *Brief Funct. Genomic. Proteomic* 2006;5:154–168. [PubMed: 16798750]
53. Zimmer JS, Monroe ME, Qian WJ, Smith RD. Advances in proteomics data analysis and display using an accurate mass and time tag approach. *Mass Spectrom. Rev* 2006;25:450–482. [PubMed: 16429408] • Detailed description of mass spectrometry-based proteomics with special emphasis on the accurate mass and time tag approach.

54. Shen Y, Zhang R, Moore RJ, et al. Automated 20 kpsi RPLC-MS and MS/MS with chromatographic peak capacities of 1000–1500 and capabilities in proteomics and metabolomics. *Anal. Chem* 2005;77:3090–3100. [PubMed: 15889897]
55. Adkins JN, Mottaz HM, Norbeck AD, et al. Analysis of the *Salmonella* Typhimurium proteome through environmental response toward infectious conditions. *Mol. Cell. Proteomics* 2006;5:1450–1461. [PubMed: 16684765]
56. Beuzon CR, Banks G, Deiwick J, Hensel M, Holden DW. pH-dependent secretion of SseB, a product of the SPI-2 type III secretion system of *Salmonella* Typhimurium. *Mol. Microbiol* 1999;33:806–816. [PubMed: 10447889]
57. Manes NP, Gustin JK, Rue J, et al. Targeted protein degradation by *Salmonella* under phagosome-mimicking culture conditions investigated using comparative peptidomics. *Mol. Cell. Proteomics* 2007;6:717–727.727 [PubMed: 17228056] • Analysis of the degraded and small proteins present in *Salmonella*.
58. Coldham NG, Woodward MJ. Characterization of the *Salmonella* Typhimurium proteome by semi-automated two dimensional HPLC-mass spectrometry: detection of proteins implicated in multiple antibiotic resistance. *J. Proteome Res* 2004;3:595–603. [PubMed: 15253442]
59. Ansong C, Yoon H, Norbeck AD, et al. Proteomics analysis of the causative agent of typhoid fever. *J. Proteome Res* 2008;7:546–557. [PubMed: 18166006]
60. Guerrero C, Tagwerker C, Kaiser P, Huang L. An integrated mass spectrometry-based proteomic approach: quantitative analysis of tandem affinity-purified *in vivo* cross-linked protein complexes (QTAX) to decipher the 26 S proteasome-interacting network. *Mol. Cell. Proteomics* 2006;5:366–378.378 [PubMed: 16284124] •• Useful description of how to use crosslinking with proteomics methods to elucidate protein–protein interactions.
61. Tagwerker C, Flick K, Cui M, et al. A tandem affinity tag for two-step purification under fully denaturing conditions: application in ubiquitin profiling and protein complex identification combined with *in vivo* cross-linking. *Mol. Cell. Proteomics* 2006;5:737–748. [PubMed: 16432255]
62. Sutherland BW, Toews J, Kast J. Utility of formaldehyde cross-linking and mass spectrometry in the study of protein-protein interactions. *J. Mass Spectrom* 2008;43:699–715. [PubMed: 18438963]
63. Bijlsma JJ, Groisman EA. The PhoP/PhoQ system controls the intramacrophage type three secretion system of *Salmonella enterica*. *Mol. Microbiol* 2005;57:85–96. [PubMed: 15948951]
64. Rinner O, Seebacher J, Walzthoeni T, et al. Identification of cross-linked peptides from large sequence databases. *Nat. Methods* 2008;5:315–318. [PubMed: 18327264]
65. Rogers LD, Kristensen AR, Boyle EC, et al. Identification of cognate host targets and specific ubiquitylation sites on the *Salmonella* SPI-1 effector SopB/SigD. *J. Proteomics* 2008;71:97–108.108 [PubMed: 18541478] • An example of how stable isotope labeling with amino acids in cell culture (SILAC) can be used to overcome special challenges in proteomics.
66. Nystrom T. Stationary-phase physiology. *Annu. Rev. Microbiol* 2004;58:161–181. [PubMed: 15487934]
67. Putsep K, Branden CI, Boman HG, Normark S. Antibacterial peptide from *H. pylori*. *Nature* 1999;398:671–672. [PubMed: 10227288]
68. Ahmer BM. Cell-to-cell signalling in *Escherichia coli* and *Salmonella enterica*. *Mol. Microbiol* 2004;52:933–945. [PubMed: 15130116]
69. Oldstone MB. Molecular mimicry, microbial infection, and autoimmune disease: evolution of the concept. *Curr. Top. Microbiol. Immunol* 2005;296:1–17. [PubMed: 16329189]
70. Rytönen A, Poh J, Garmendia J, et al. SseL, a *Salmonella* deubiquitinase required for macrophage killing and virulence. *Proc. Natl Acad. Sci. USA* 2007;104:3502–3507.3507 [PubMed: 17360673] • Describes the mechanism by which one of the many *Salmonella* secreted effectors (perhaps 100) alter the host cell environment by encoding specific enzymatic activities directed at a single or subset of proteins. Of the 35 known *Salmonella* secreted effectors a defined function has been assigned to fewer than ten.
71. Mazurkiewicz P, Thomas J. Thompson *et al.*: SpvC is a *Salmonella* effector with phosphothreonine lyase activity on host mitogen-activated protein kinases. *Mol. Microbiol* 2008;67:1371–1383.1383 [PubMed: 18284579] • Describes the mechanism by which one of the many *Salmonella* secreted

effectors alter the host cell environment by encoding specific enzymatic activities directed at a single or subset of proteins.

72. Patel JC, Galan JE. Investigating the function of Rho family GTPases during *Salmonella* host cell interactions. *Methods Enzymol* 2008;439:145–158. [PubMed: 18374162]
73. Becker D, Selbath M, Rollenhagen C, et al. Robust *Salmonella* metabolism limits possibilities for new antimicrobials. *Nature* 2006;440:303–307. [PubMed: 16541065]

Websites

101. Centers for Disease Control and Prevention. Spotlight: *Salmonella* saintpaul outbreak investigation. 2008. www.cdc.gov/Salmonella
102. Primary Data. Published and some unpublished data generated at Pacific Northwest National Laboratory discussed in this text. <http://omics.pnl.gov>
103. Resource Center for Biodefense Proteomics Research. <http://proteomicsresource.org>

Figure 1. The intracellular pathogen *Salmonella* subverts the host cell's defense by secretion of virulence factors directly to the host cell cytoplasm.

As an example, this is a micrograph of a *Salmonella*-infected monocyte/macrophage cell (red; [20]). The secreted protein (srfH) is fused to an enzyme that changes the fluorescence of the infected cell from green to blue. The two small cells are T lymphocytes and contain the fluorescent dye coumarin cephalosporin fluorescein (green; [17]). When cleaved by β -lactamase the dye no-longer displays Forster resonance energy transfer altering the emission spectrum to blue ([16]; courtesy of Cell Press).