Analysis of the *Salmonella typhimurium* Proteome through Environmental Response toward Infectious Conditions*

Joshua N. Adkins‡, Heather M. Mottaz§, Angela D. Norbeck‡, Jean K. Gustin¶, Joanne Rue¶, Therese R. W. Clauss‡, Samuel O. Purvine§, Karin D. Rodland‡, Fred Heffron¶, and Richard D. Smith‡‖

Salmonella enterica **serovar Typhimurium (also known as *Salmonella typhimurium*) is a facultative intracellular pathogen that causes ~8,000 reported cases of acute gastroenteritis and diarrhea each year in the United States. Although many successful physiological, biochemical, and genetic approaches have been taken 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. We used a liquid chromatography-mass spectrometry-based "bottom-up" proteomic approach to generate a more complete picture of the gene products that *S. typhimurium* synthesizes under typical laboratory conditions as well as in culture media that are known to induce expression of virulence genes. When grown to logarithmic phase in rich medium, *S. typhimurium* is known to express many genes that are required for invasion of epithelial cells. Conversely stationary phase cultures of *S. typhimurium* express genes that are needed for both systemic infection and growth within infected macrophages. Lastly bacteria grown in an acidic, magnesium-depleted minimal medium (MgM) designed to mimic the phagocytic vacuole have been shown to up-regulate virulence gene expression. Initial comparisons of protein abundances from bacteria grown under each of these conditions indicated that the majority of proteins do not change significantly. However, we observed subsets of proteins whose expression was largely restricted to one of the three culture conditions. For example, cells grown in MgM had a higher abundance of Mg^{2+} transport proteins than found in other growth conditions. A second more virulent *S. typhimurium* strain (14028) was also cultured under these same growth conditions, and the results were directly compared with those obtained for strain LT2. This comparison offered a unique opportunity to contrast protein populations in these closely related bacteria. 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. These *pdu* operon proteins were validated in culture and during macrophage infection. Our work provides further support for earlier observations that suggest *pdu* gene expression contributes to *S. typhimurium* pathogenesis. *Molecular & Cellular Proteomics 5:1450–1461, 2006.***

Salmonella enterica serovar Typhimurium (*Salmonella typhimurium*) infection is one of the leading causes of salmonellosis, a severe form of food poisoning; ~8,000 cases are reported yearly in the United States alone (1). *S. typhimurium* infections can be life-threatening in elderly, very young, and immunocompromised patients. This bacterium is closely related to *Salmonella typhi*, the causative agent of typhoid fever, which results in ~600,000 deaths worldwide per year and is a serious concern for travelers and domestic populations in Africa, South America, Mexico, and parts of Asia (2). Globally the incidence of typhoid fever is decreasing; however, nontyphoidal salmonellosis (*S. typhimurium*) is increasing (3). Because of the increasing prevalence of antibiotic-resistant *S. typhimurium* isolates, an understanding of the growth and pathogenesis of this organism is important for developing new therapeutic agents (4–6).

S. typhimurium infection and pathogenicity are complex, highly integrated processes that cannot be attributed to any single protein activity. Within a well adapted pathogen, critical pathogenesis functions may have redundant or alternative pathways. Many studies have used biologically relevant models of infection to analyze the molecular basis of infection (7–9). These studies have shown that systemic infection of animals is largely enabled through a type III secretion system (TTSS)[1] encoded by *Salmonella* pathogenicity island (SPI) 2. Proteins secreted by

From the ‡Biological Science Division and §Environmental Molecular Science Laboratory, Pacific Northwest National Laboratory, Richland, Washington 99352 and ¶Department of Molecular Microbiology and Immunology, Oregon Health and Sciences University, Portland, Oregon 97239

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[1] The abbreviations used are: TTSS, type III secretion system; SPI, *Salmonella* pathogenicity island; Pdu, propanediol utilization; log, logarithmic; MgM, acidic, magnesium-depleted minimal medium; LB, Luria Bertani; HA, hemagglutinin; BCA, bicinchoninic acid; SPE, solid phase extraction; SCX, strong cation exchange; ABC, ATP-binding cassette.

the SPI2 TTSS allow *Salmonella* to grow within and eventually kill host macrophages, thereby overcoming a critical component of the innate immune response. This ability is an essential prerequisite for successful systemic infection. Additional *Salmonellae* genomic islands have been identified, and in some cases these islands are also required for virulence (10, 11). Many other virulence genes are dispersed throughout the *Salmonella* genome, although their roles have not yet been clearly defined (12). Proteomic profiling, as performed in other bacterial systems, is a useful approach for obtaining a global overview of the proteins present in a system under differing conditions (13–15) and can aid in understanding the molecular determinants involved with pathogenesis. This understanding is essential for developing effective strategies to combat infection as well as for revealing new therapeutic targets (16).

To evaluate the *S. typhimurium* proteome, we used a "bottom-up" proteomic approach that utilizes mass spectrometry to identify peptides derived from enzymatically digested proteins. We concentrated primarily on *S. typhimurium* LT2, a sequenced laboratory-derived strain that harbors a defective *rpoS* gene (17). The *rpoS* gene codes for an alternative sigma factor (σ^S) that has been shown to be critical for the expression of many virulence-related proteins (17, 18). Relative changes in protein abundance were examined across three growth conditions. Logarithmic (log) and stationary phase cultures were chosen because these represent the two most well characterized hallmarks of the bacterial growth curve. At the same time, log phase *S. typhimurium* cultures express components of SPI1 that are required for invasion of host cells. Likewise stationary phase cultures have been shown to express components of SPI2 that are required for successful systemic infection. Lastly either growth in or brief exposure to an acidic, magnesium-depleted minimal medium (MgM) has been shown to approximate the environment found within the *Salmonella*-containing vacuoles that are observed in infected host macrophages. Earlier studies have noted that low pH and low Mg^{2+} are important signals that induce expression and assembly of the SPI2-encoded TTSS as well as other proteins related to macrophage infection (19–27). These three growth conditions allowed us to gather data from standard well characterized culture conditions as well as from conditions that are known to induce expression of virulence determinants. A similar dataset was then generated for the more virulent *S. typhimurium* strain ATCC 14028, which was compared with the LT2 dataset to identify those proteins responsible for different virulence phenotypes. Our data indicate that such a comparative proteomic approach provides a comprehensive view of protein abundances as they vary with respect to time, environment, and genotype, which can simultaneously lead to the identification of putative virulence factors.

MATERIALS AND METHODS

Reagents—The following reagents were used in proteomic sample preparation: Nanopure or Milli-Q quality water (~18 megohm·cm or better), ammonium bicarbonate (NH_4HCO_3), bicinchoninic acid (BCA) or Coomassie protein assay reagents (Pierce), urea, thiourea, DTT, CHAPS, Rapigest™ surfactant (Waters), calcium chloride, sequencing-grade modified trypsin (Promega), HPLC-grade methanol (MeOH), TFA, ACN, ammonium formate, formic acid, and ammonium hydroxide (NH_4OH). All reagents were obtained from Sigma unless otherwise specified.

Bacterial Growth—*S. typhimurium* strains 14028 and LT2 were grown and harvested using standard batch culture procedures (28). Cells from a single colony were inoculated into 5 ml of Luria Bertani (LB) medium and then grown for 16 h with shaking at 37 °C. This starter culture was then diluted 1:100 into 300 ml of fresh LB medium in a 2-liter Erlenmeyer flask and grown for 16 h with shaking (200 rpm) at 37 °C. Once the log and stationary phase cultures reached an A_{600} of 0.6 and 2.0, respectively, the cells were harvested via centrifugation at 4,000 × *g*. For the MgM cultures, cells were first grown in LB medium to stationary phase as described above, rinsed twice with MgM (29), resuspended in an equal volume of MgM, and then incubated at 37 °C with shaking (200 rpm) for 4 h. The cells were harvested as described above. After harvesting the cells for each culture condition, the cell pellets were washed twice with Cellgro Dulbecco's PBS (Mediatech) and finally pelleted in microcentrifuge tubes to an approximate wet weight of 0.1 g/tube. The cells were frozen and stored at −80 °C until needed.

Strain Construction—Single copy, chromosomal hemagglutinin (HA)-tagged variants of the *S. typhimurium pduB*, *pduC*, and *pduO* genes were created using the PCR-based method described by Uzzau *et al.* (30). In the first step of this procedure, homologous recombination was used to place both an HA tag at the 3′-end and a kanamycin resistance determinant immediately downstream of each gene to allow for direct selection of recombinants. Diagnostic PCR was then performed to confirm the resulting recombinants. The presence of FRT sites (FLP recombinase recognition targets) that flanked the kanamycin cassette permitted the subsequent removal of the cassette. This step was performed to minimize polar effects on downstream genes and was accomplished by transforming each mutant with a plasmid that encodes the FLP recombinase (pCP20). The resulting strains were tested for loss of the kanamycin marker and then subjected to a final round of diagnostic PCR.

Bacterial Infection of Cultured Macrophages—J774 and RAWS macrophage-like cell lines were plated in 6-well plates at ~2.4×10^6 cells/well and incubated overnight at 37 °C with 5% CO_2. The next day, stationary phase bacteria were added at a multiplicity of infection of 50. Infections were initiated by centrifuging the bacteria onto the cell monolayers at 1,000 × *g* for 5 min and then incubated at 37 °C with 5% CO_2 for 30 min. To remove extracellular bacteria after the infection, the monolayers were washed twice with PBS, Dulbecco's modified Eagle's medium plus gentamicin (100 μg/ml final concentration), and the cells were incubated for 1 h. The presence of gentamicin eliminates all extracellular bacteria. After that time, the cells were washed twice with PBS, overlaid with Dulbecco's modified Eagle's medium plus gentamicin (10 μg/ml final concentration), and incubated at 37 °C with 5% CO_2 for the remainder of the experiment.

Preparation of Host Cell Lysates—At various times postinfection, the infected monolayers were washed once with PBS, 1 ml of ice-cold PBS was added to each well, and the cells were removed from the plates with a cell scraper. The contents of each well were then transferred to 1.6-ml Eppendorf tubes and centrifuged at 500 × *g* for 5 min.

The supernatants were removed, and the cell pellet was resuspended in lysis buffer (50 mM HEPES, 1 mM EDTA, 1 mM EGTA, 1% Triton, 100 mM PMSF, protease inhibitor mixture (Roche Applied Science), 50 mM NaF, and 2 mM sodium orthovanadate). The lysates were incubated on ice for 30 min and then centrifuged for 10 min at

10,000 × *g*. The supernatants were transferred to fresh tubes (host cytosolic fraction), and the pellets were immediately resuspended in 1× Laemmli sample buffer (bacterial cell fraction). BCA assays were performed for all host cytosolic samples, and equal amounts of protein were subjected to immunoblot analysis as described above. For the bacterial cell fractions, equal volumes of each sample were used for immunoblot analyses.

Immunoblot Analysis—The *pduB*::HA, *pduC*::HA, and *pduO*::HA strains were each cultured under the same three conditions (LB logarithmic, LB stationary, and MgM) described for our initial proteomic characterization of strains LT2 and 14028, and the final OD (600 nm) of each culture was recorded. The cells from 1 ml of each culture were harvested by centrifugation at 10,000 × *g* in a microcentrifuge at 4 °C, washed twice with an equal volume of ice-cold Dulbecco's PBS, resuspended in 100 μl of Laemmli 1× sample buffer, and boiled for 5 min. Protein samples were normalized to culture A_{600} and loaded into the wells of 12% Tris-Cl SDS-PAGE gels (Invitrogen). After separating the proteins on the gel, the proteins were electrophoretically transferred to PVDF membranes (Millipore). The membranes were blocked in TBS plus 5% powdered nonfat dry milk for 1 h, probed with an anti-HA monoclonal antibody (Covance, 1:1,000, in block solution) for 1 h, washed 3 × 5 min with TBS, probed with a sheep-anti-mouse secondary antibody (Amersham Biosciences, in block solution) for 30 min, and finally washed 3 × 5 min with TBS. The immune complexes were detected via chemiluminescence by using PerkinElmer Life Sciences "Western Lightning" reagents and then exposed to XAR Biofilm (Eastman Kodak Co.).

Global and Soluble Protein Preparation—Cell pellets were resuspended in 100 mM NH_4HCO_3, pH 8.4 buffer and lysed by using 0.1 mM zirconia/silica beads in a 2.0-ml Cryovial with vigorous vortexing for a total of 3 min with cooling steps. The supernatant and subsequent washes were transferred from the beads into new Cryovials. The beads were repeatedly washed until the supernatant was clear.

After protein concentration was determined for the samples, urea and thiourea were added to final concentrations of 7 and 2 M, respectively. Following addition of DTT (5 mM), the samples were incubated at 60 °C for 30 min. The samples were then diluted 10-fold with buffer, and $CaCl_2$ was added (1 mM) followed by trypsin in a 1:50 trypsin:protein ratio. The samples were digested for 3 h at 37 °C and subsequently cleaned using a C_{18} solid phase extraction (SPE) column (Supelco). Each 1-ml 100- or 50-mg SPE column was conditioned with MeOH and rinsed with 0.1% TFA in water. Samples were introduced to the columns and then washed with 95:5 H_2O:ACN that contained 0.1% TFA. Excess liquid was removed from the columns under vacuum, and the samples were eluted with 80:20 ACN:H_2O that contained 0.1% TFA and concentrated in a SpeedVac (Thermo-Savant) to a final volume of ~100 μl. A BCA protein assay was performed to determine peptide concentrations prior to analysis.

Insoluble Protein Preparation—Cell pellets were treated and lysed as described above for global and soluble protein preparations. The lysate was centrifuged at 1,300 × *g* at 4 °C for 2 min, and the supernatant was transferred to polycarbonate ultracentrifuge tubes (Beckman) and centrifuged at 4 °C at 356,000 × *g* for 10 min. Pellets were resuspended in 50 mM NH_4HCO_3, pH 7.8, and ultracentrifuged under the same conditions as used in the previous step. A BCA protein assay (31) was performed on the pellets resuspended in water, and the samples were ultracentrifuged once again (as described above) before discarding the supernatant. Pellets were resuspended in ~200 μl of a solubilization solution (7 M urea, 2 M thiourea, 1% CHAPS in 50 mM ammonium bicarbonate, pH 7.8), and DTT was added to a final concentration of 9.7 mM. Samples were incubated at 60 °C for 30 min, then diluted, and digested in the same manner as described for the global and soluble protein preparation. Samples were cleaned by using an appropriately sized strong cation exchange (SCX) SPE column (Supelco). Each 1-ml 100-mg column was conditioned with MeOH, rinsed in varying sequences and amounts with 10 mM ammonium formate in 25% ACN, pH 3.0; 500 mM ammonium formate in 25% ACN; and Nanopure water. Samples were acidified to a pH of ≤4.0 by adding 20% formic acid followed by centrifugation at 16,000 × *g* for 5 min. Samples were then introduced to the columns and washed with 10 mM ammonium formate in 25% ACN, pH 3.0. Excess liquid was removed from the columns under vacuum. The samples were eluted with 80:15:5 MeOH:H_2O:NH_4OH and concentrated to ~100 μl using a SpeedVac. Final peptide concentrations were determined using a BCA protein assay.

Rapigest Preparation—Sample preparation with Rapigest followed the protocol outlined in the instructions from Waters with the exception of using 2.0% TFA instead of HCl to reduce the sample pH to 3.0 and incubating for 60 min at 37 °C to precipitate the surfactant. After digestion and acidification, the samples were centrifuged, and the supernatant was transferred to a clean microcentrifuge tube. The sample pH was increased to ~7 with NH_4OH, and the peptide concentration was determined using a BCA protein assay.

SCX Fractionation—SCX fractionation was performed as described previously (32). A brief description of the approach and modifications to the method are summarized here. The peptides were resuspended in 900 μl of mobile phase A and separated on an Agilent 1100 system (Agilent, Palo Alto, CA) that was equipped with a quaternary pump, degasser, diode array detector, and Peltier-cooled autosampler and fraction collector (both set at 4 °C). The mobile phases consisted of 10 mM ammonium formate, 25% ACN, pH 3.0 (A) and 500 mM ammonium formate, 25% ACN, pH 6.8 (B). The gradient consisted of maintaining mobile phase A at 100% for the first 10 min and then increasing mobile phase B from 0 to 50% over the next 40 min and from 50 to 100% over the following 10 min before sustaining 100% mobile phase B for a final 10 min. A flow rate of 0.2 ml min^{-1} was maintained throughout the gradient. Spectra were obtained at 280 nm, and fractions were collected over the initial 70 min of the gradient.

Capillary Liquid Chromatography-Mass Spectrometry Analysis—The HPLC system consisted of two 100-ml Isco Model 100DM syringe pumps and Series D controller (Isco, Inc., Lincoln, NE); an in-house manufactured stir bar style mobile phase mixer (2.5-ml volume); two four-port, two-position Valco valves (Valco Instruments Co., Houston, TX) for mobile phase and capillary column selection; and a six-port, two-position Valco valve that was equipped with a 10-μl sample loop for manual injections. The mixer and valves were mounted on an in-house manufactured rack assembly that was custom fit to a PAL autosampler (Leap Technologies, Carrboro, NC) for unattended routine analysis. Reversed-phase capillary HPLC columns were manufactured in-house by slurry-packing 5-μm Jupiter C_{18} stationary phase (Phenomenex, Torrence, CA) into a 60-cm length of 360-μm-outer diameter × 150-μm-inner diameter fused silica capillary tubing (Polymicro Technologies Inc., Phoenix, AZ) that incorporated a 2-μm retaining screen in a 0.005-inch capillary-bore union (Valco Instruments Co., Houston, TX).

The mobile phase consisted of 0.2% acetic acid and 0.05% TFA in water (A) and 0.1% TFA in 90% acetonitrile, 10%water (B). The mobile phase was degassed by using an in-line Alltech vacuum degasser (Alltech Associates, Inc., Deerfield, IL). The HPLC system was equilibrated at 5,000 p.s.i. with 100% mobile phase A, and then the mobile phase selection valve was switched 20 min after injection from position A to B, which created an exponential gradient as mobile phase B displaced A in the mixer. An approximately ~5-cm length of 360-μm-inner diameter fused silica tubing packed with 5-μm C_{18} particles was used to split ~25 μl/min flow before it reached the injection valve. The split flow controlled the gradient speed under conditions of constant pressure operation. Flow through the capillary HPLC column when equilibrated to 100% mobile phase A was ~2 μl/min.

TABLE I

S. typhimurium peptides observed for each method

Totals for each method preparation are listed in bold in the corresponding row. Counts and percentages of peptides shared between the methods are shown. (percentages are derived by dividing the value by the maximum value for that row). Total peptides that are only found in one method are listed in the farthest right column. The aggregate values are shown for the different culture methods, but the trends held when individual conditions were analyzed. Therefore, they constituted pseudoreplicate analyses.

	Peptide list				
	Global	Soluble	Insoluble	Rapigest	Unique to method
Global	**24,511**	18,102 (74%)	10,426 (43%)	15,261 (62%)	3,517
Soluble	18,102 (72%)	**25,153**	9,572 (38%)	14,868 (59%)	4,726
Insoluble	10,426 (62%)	9,572 (57%)	**16,756**	12,544 (75%)	2,863
Rapigest	15,261 (62%)	14,868 (60%)	12,544 (51%)	**24,717**	4,943
Total					**41,880**

MS analysis was performed using a Thermo model LTQ ion trap mass spectrometer (ThermoQuest Corp., San Jose, CA) with ESI. The HPLC column was coupled to the mass spectrometer by using an in-house manufactured interface. Neither sheath gas nor makeup liquid was used. The heated capillary temperature and spray voltage were 200 °C and 2.2 kV, respectively. Data acquisition began 20 min after the sample was injected and continued for 100 min over a mass (*m/z*) range of 400–2,000. For each cycle, the five most abundant ions from MS analysis were selected for MS/MS analysis using a collision energy setting of 45%. A dynamic exclusion time of 60 s was used to discriminate against previously analyzed ions.

Data Analysis—Peptides were identified by using SEQUEST™ to search the mass spectra from 300 LC-MS/MS analyses. The searches were performed using the annotated *S. typhimurium* fasta data file, containing 4,550 protein sequences provided by The Institute for Genomic Research (www.tigr.org/, September 19, 2004, Stanford University), a standard parameter file with no modifications to amino acid residues and a mass error window of 3 *m/z* units for precursor mass and 0 *m/z* units for fragmentation mass. The searches were allowed for all possible peptide termini, *i.e.* not limited by tryptic only termini. The large sets of tentative peptide identifications were subsequently combined and binned by condition and strain.

These results were filtered using criteria established by Washburn *et al.* (33), and a discriminant approach (34) with a score of at least 0.9 was used to increase confidence in identified peptides. The discriminant score is representative of the quality of the SEQUEST identification and is based on a combination of XCorr, ΔCn, tryptic state, and the difference between the observed elution time and the predicted elution time for the peptide sequence (34, 35). An estimate of the false positive rate was determined by using a method described elsewhere (36) and a subset of 60 analyses. A calculated false discovery rate of 0.06% was determined by using the combined filtering rules (*i.e.* 0.9% for a discriminant score of 0.9 only and 1.8% for SEQUEST filtering only). The number of peptide observations from each protein was used as a rough measure of relative abundance; no values called out in subsequent analyses differed by less than 3-fold between any two conditions. Multiple charge states of a single peptide were considered as individual observations as were the same peptides detected in different mass spectral analyses. Similar approaches for quantitation have been described previously (32, 37–42).

To address concerns as to whether the growth medium contributed to the *Salmonella* proteome, SEQUEST searches were performed with the same subset of 60 analyses as stated above but with a non-redundant protein data file, containing 283,416 protein sequences, available from the Protein Information Resource (pir.georgetown.edu as Protein Sequence Database release 78.00). This search indicated 94% of the peptides were directly attributable to a *Salmonella* protein sequence. Of the 6% of peptides not directly attributable to *Salmonella* proteins, the vast majority (>90%) were typically abundant proteins (*e.g.* ribosomal and RNA polymerase subunits) derived from related bacterium such as *Escherichia*, *Yersinia*, *Pseudomonas*, *Haemophilus*, *Vibrio*, and *Brucella*. The identification of such peptides may represent differences between the annotation of the LT2 genome and the true peptide content of both the LT2 and 14028 proteomes. This speculation will need to be studied in greater detail once a version of the 14028 protein data file is made available. We also detected ~0.1% yeast peptides (similar in magnitude to the false discovery rate suggested above), which illustrates a limited contamination by the yeast extract present in the LB medium. Lastly 0.5% of the peptides were identified as contamination from the protein standards that were used for instrument quality assurance/quality control (43).

Proteins identifications were required to have at least three peptide observations that met our SEQUEST and discriminant criteria. To compare proteins, the peptide observation counts for each protein were first normalized by summing across all comparative states, a process whereby the observed counts for a particular protein for all conditions were first summed, and then the count for each condition was divided by that sum value. An arbitrary value of 1 was supplied for missing data points (*i.e.* proteins not observed in a specific condition). The effect of this procedure was to normalize protein measurements to a similar magnitude regardless of the total number of analyses and the total number of peptide identifications. Due to the assignment of 1 for missing data, those proteins with few total observations (*i.e.* peptides detected) were less likely to show significant differences between conditions.

Final results were subjected to K-means cluster analysis using OmniViz™ 3.8 (OmniViz, Maynard, MA) (44) with a correlation by magnitude and shape. OmniViz 3.8 was then used to query the results for differences among growth conditions and strains.

RESULTS

Sample Preparation Method Comparison—Four sample preparation methods applicable to peptide level bottom-up proteomics, 1) global tryptic digest on whole-cell lysates, 2) soluble preparation, 3) insoluble preparation on the pellet remaining from the soluble protein separation, and 4) commercially available Rapigest, were compared to determine the optimal method for attaining comprehensive proteomic coverage with minimal sample manipulation. Unique peptide sequences observed for each method in terms of both counts and percentages of peptides shared among the methods are presented in Table I. Samples prepared by using the global tryptic digest method yielded a total of 24,511 unique peptide sequences of which 3,517 were found solely with this method

TABLE II

S. typhimurium proteins observed for each method

Totals for each method preparation are listed in bold in the corresponding row. Counts and percentages of proteins shared between the methods are shown (percentaages are derived by dividing the value by the maximum value for that row). Total peptides that are only found in one method are listed in the farthest right column. The aggregate values are shown for the different culture methods, but the trends held when individual conditions were analyzed. Therefore, they constituted pseudoreplicate analyses.

	Protein list				
	Global	Soluble	Insoluble	Rapigest	Unique to method
Global	**1,999**	1,802 (90%)	1,609 (81%)	1,847 (92%)	12
Soluble	1,802 (89%)	**2,030**	1,501 (74%)	1,804 (89%)	67
Insoluble	1,609 (86%)	1,501 (81%)	**1,864**	1,661 (89%)	97
Rapigest	1,847 (87%)	1,804 (85%)	1,661 (78%)	**2,121**	63
Total					**2,343**

of preparation. Approximately 74% of these 24,511 sequences were also observed in the soluble preparation samples, whereas 62% of these sequences were shared with the Rapigest preparation. The insoluble preparations had the fewest number of peptides shared with the global preparation (43%). Samples prepared by using the soluble preparation method yielded a total of 25,153 peptides of which 4,726 were unique to this method. A majority of the soluble peptides (72%) were also observed in samples prepared with the global method. Not unexpectedly, the soluble preparation had the least overlap with the insoluble preparation (38%); however, a considerable portion of the soluble peptides were shared with Rapigest (59%). The insoluble preparation yielded a total of 16,756 peptides of which 2,863 were unique to this method of preparation. Of these, 62% were observed in the global tryptic digest, 57% were observed in the soluble preparation, and 75% were observed in the Rapigest method. The Rapigest protocol resulted in a high number of shared peptide observations for both global and soluble sample preparation methods, and this method also observed the highest number of shared peptides with the insoluble preparation compared with the other two methods.

Table II shows the number of proteins observed for each sample preparation method in terms of both counts and percentages of proteins shared among the methods for comparison with the peptide identification observations in Table I. A minimum of three filter-passing peptides within one condition was required for each protein reported, thus providing an extremely high level of confidence for all proteins identified. The global preparation samples yielded a total of 1,999 proteins of which 12 were unique to that method of preparation. The soluble preparation had 2,030 proteins of which 67 were found solely with this method. A large majority of the soluble proteins (89%) were also detected in the global and Rapigest preparations, whereas the insoluble preparation had the least in common with these two preparations (74%). The insoluble preparation resulted in identification of 1,864 total proteins of which 97 proteins were unique to this method of preparation. Of these 1,864 proteins, 86% were also observed in samples prepared using the global tryptic digest, 81% were observed in the soluble preparation, and 89% were observed in the Rapigest method.

On the basis of these results and because of its simple protocol and broad protein type detection, the Rapigest sample preparation method was selected as the preferred method for *S. typhimurium* global proteome analysis. The insoluble method was considered a good complement to the Rapigest method due to its higher specificity for membrane proteins than any other method.

Functional Analysis of Identified Proteins—Table III shows that the identified proteins represent a general subset of proteins from the genetic translation with no apparent biases toward a specific main role category. Many (17%) of these proteins are classified as unknown or unclassified by function (Table III). Additionally 387 of the 2,343 total proteins are thought to be involved in energy metabolism, and another 8.5% (224) are involved with transport and binding, although they are underrepresented compared with the entire genome. Unlike other categories, the proteins observed in the mobile or extrachromosomal elements are underrepresented in our dataset relative to their prevalence in the genome.

Growth Condition Comparisons—In general, the three growth conditions (log phase, stationary phase, and MgM growth) had very similar protein compositions. Of the 2,343 proteins identified from the combined results, 1,589 proteins were detected in all conditions, and 1,995 proteins were detected in at least two conditions. Because of the size and approximate similarity of the proteomes in these growth conditions, data mining and visualization techniques were used to discern the most significant differences among conditions as well as to determine a qualitative measure of confidence.

The use of data mining techniques proved to be useful for identifying some trends between culture conditions. A heat map (the result of a hierarchical clustering of protein observations) was generated to visually highlight proteins that were observed nearly exclusively in a single condition (Fig. 1). These proteins most likely represent the best opportunity for studying the different growth conditions. The 115 proteins (from two clusters) that were both uniquely and highly expressed for the MgM growth condition included multiple Mg^{2+} transport (MgtA, MgtB, and MgtC), multiple propanediol utilization (Pdu proteins), and ABC superfamily proteins (ArtJ, CycA, CysP, and ModF). Additionally a sensor

TABLE III

The Institute for Genomic Research "functional" categories for the entire annotated genome and characterized proteome

Shaded cells represent the two extremes between the observed proteome fraction and the annotated genome. Some proteins are annotated to multiple categories, accounting for the larger total protein count in the pie chart.

Functional Categories	Total from Genome	Percent of Genome	Total of Observed Proteome	Percent of Observed Proteome	Observed Fraction of Category
Amino acid biosynthesis	130	2.6%	101	3.8%	0.78
Biosynthesis of cofactors, prosthetic groups, and carriers	167	3.3%	123	4.7%	0.74
Cell envelope	479	9.4%	190	7.2%	0.40
Cellular processes	289	5.7%	169	6.4%	0.58
Central intermediary metabolism	170	3.3%	114	4.3%	0.67
DNA metabolism	166	3.3%	116	4.4%	0.70
Energy metabolism	601	11.8%	387	14.7%	0.64
Fatty acid and phospholipids metabolism	80	1.6%	59	2.2%	0.78
Hypothetical proteins	78	1.5%	47	1.8%	0.60
Mobile and extrachromosomal element functions	250	4.9%	23	0.9%	0.09
Protein fate	191	3.8%	134	5.1%	0.70
Protein synthesis	375	7.4%	207	7.8%	0.55
Purines, pyrimidines, nucleosides, and nucleotides	81	1.6%	68	2.6%	0.84
Regulatory functions	305	6%	153	5.8%	0.50
Signal transduction	26	0.5%	11	0.4%	0.42
Transcription	57	1.1%	46	1.7%	0.81
Transport and Binding proteins	628	12.3%	224	8.5%	0.36
Unclassified or unknown	1016	20%	466	17.7%	0.46

kinase in a two-component regulatory system (PhoR) was also observed to be highly abundant and uniquely present in the MgM growth condition. There were 40 proteins (from one cluster) that were uniquely expressed in the log growth condition. Most of these proteins, *e.g.* cysteine sulfinate desulfinase (DeaD) and a protein associated with glucose-inhibited division (GidA), did not appear to be functionally related with the exception of multiple flagellar biosynthesis proteins (FlgD, FliA, FliZ, FliS, and FliT). Notably Pdu and ABC superfamily proteins were not seen in the log growth cluster. Stationary growth proteins (23 from one cluster) included known host cell invasion proteins (SipA, SipB, SipC, and SipD). Ribosome

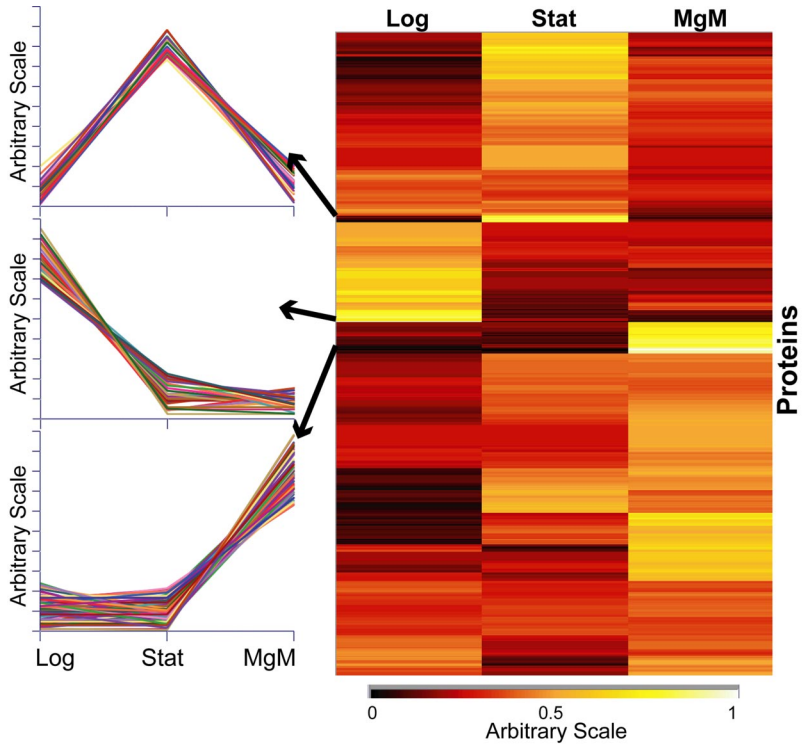

FIG. 1. **Heat map representation of the proteins found within each growth condition.** Observation counts of the peptides for each protein are used to represent a relative protein quantitation. The observation counts were normalized by dividing each protein value by the sum of values of that protein row, and the scale from least abundant to highest ranges from 0 to 1 and *black* to *yellow*. The *first column* (*C*) shows the groups of proteins formed through heirarchial cluster analysis, and the columns in the heat map represent the growth conditions, log phase (*Log*), stationary phase (*Stat*), and MgM growth (*MgM*). Specific clusters of proteins that were nearly unique in each of the three conditions were lineplotted along the *left side*. (See the supplemental data.)

modulation factor (Rmf), a key indicator of the stationary growth condition, was also present in the stationary cluster.

Comparison of S. typhimurium Strains 14028 and LT2—LT2 is known to harbor an *rpoS* mutation that is responsible for, among other things, virulence attenuation. The more virulent *S. typhimurium* strain 14028 possesses a wild-type copy of *rpoS*. Because the study of a more virulent strain may offer clues to the functional basis for this increased virulence, a proteomic analysis of strain 14028 was conducted in parallel with the LT2 work (17). The results indicated that for each growth condition, there was a difference in the number of peptides that are unique to each strain (Table IV). There were 2,296 proteins from strain 14028 and 2,121 proteins from the LT2 strain that were identified globally by three or more filter-passing peptide observations. Table V shows that, for most conditions, 90% of the proteins identified were common to both strains. However, interestingly the biggest difference in the protein overlap was observed for MgM cultures of 14028. The numbers of proteins found in both strains were 1,440 for the logarithmic growth condition, 1,552 for the stationary growth condition, and 1,664 for the MgM growth condition. The high similarity between the proteins expressed by each strain under all conditions suggests that future studies may benefit from a data file corresponding to the "core proteome" that could be use to normalize samples.

The most striking difference between the two strains was the abundance of Pdu proteins. These proteins were observed 5 times more frequently in strain 14028 than in LT2; this represents a highly significant change specific to the more virulent strain. Mutations in regulators of the propanediol utilization operon have been noted to reduce virulence in *Salmonella* strains.[2] Another interesting observation was the strain specificity of the Fels-1 prophage-encoded proteins. Although eight Fels-1 proteins were identified in LT2, these proteins were completely absent in 14028. This result was expected as the Fels-1 prophage is known to be absent in the 14028 genome.

Proteins expressed under MgM conditions in the LT2 and 14028 strains were similar (Fig. 2). There were 262 proteins specific to MgM; 22 were more highly abundant in LT2, and nine were more highly abundant in 14028. These proteins may relate to the differences in pathogenicity between the strains, but further studies are needed to determine the pathway(s) responsible for such changes.

Similar to LT2, the Mg^{2+} transport and Pdu proteins in 14028 were detected more often for the MgM growth condition (Fig. 3). The general trend observed for the ABC superfamily of proteins in LT2, *i.e.* increased in MgM, was also observed in 14028. There were 88 ABC superfamily proteins identified in total with most having a higher number of observations in MgM regardless of strain. Similarly the PhoR sensor kinase was found to be uniquely expressed in MgM cultures for both strains. OmpX was one of the few proteins that appeared strongly biased in cultures prepared with the insoluble sample preparation method. Thus, we found general agreement among most protein localizations determined from

[2] D. Parsons and F. Heffron, unpublished observation.

TABLE IV
Unique peptides by strain and condition

	Log phase		Stationary phase		MgM phase	
	Overlap	Strain-specific	Overlap	Strain-specific	Overlap	Strain-specific
Strain 14028	8,171	2,528	11,802	3,490	10,319	4,164
Strain LT2		2,718		4,409		5,066

TABLE V
Unique proteins by strain and condition

	Log phase		Stationary phase		MgM phase	
	Overlap	Strain-specific	Overlap	Strain-specific	Overlap	Strain-specific
Strain 14028	1,440	110	1,552	153	1,664	198
Strain LT2		62		61		132

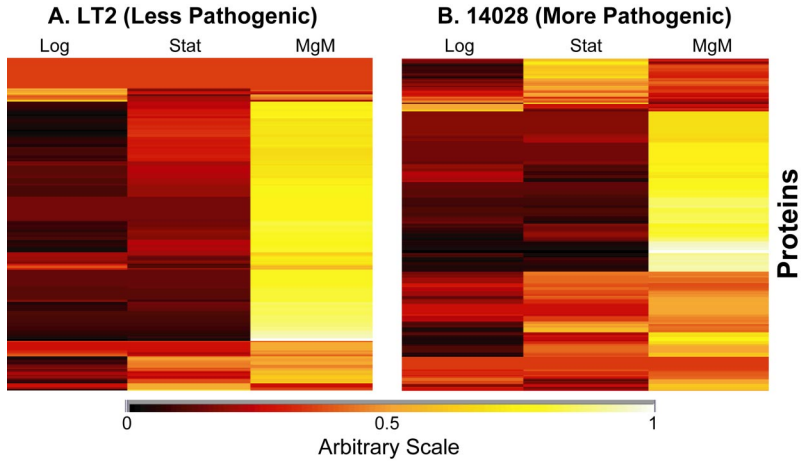

FIG. 2. **Subset of proteins that were in clusters containing proteins nearly unique in the MgM growth condition for either the LT2 or 14028 strain.** The heat map represents the observation counts by strain and condition. The observation counts were normalized such that the protein observation counts were divided by the sum for a row of protein observations, and the scale from least abundant to highest ranges from 0 to 1 and *black* to *yellow*. *A*, heat map for the LT2 strain. *B*, heat map for the 14028 strain. The figure clearly illustrates the similarity between most proteins of higher qualitative abundance in one strain under the MgM condition and the other strain under the MgM condition. The *uniformly orange-colored rows* represent proteins not observed in that specific condition, and the order of proteins is not consistent between *A* and *B*. *Stat*, stationary. (See the supplemental data.)

the more extensive sample preparation condition comparison of the LT2 strain and the more limited analysis of 14028; the exceptions may offer interesting avenues of research or highlight the value of addition sample preparation methods.

Validation of Differential Abundance and Pathogenicity of Pdu Proteins—To demonstrate the validity of our proteomic results, we opted to focus on the differential abundance of the propanediol utilization proteins. We constructed several *S. typhimurium* 14028 strains by placing a pair of tandem HA tags at the C termini of the *pduB*, *pduC*, and *pduO* genes; this allowed for subsequent immunodetection of the protein products from each of these genes. For these studies, PduB, -C, and -O were chosen because of their sizes, which are easily detected on standard protein gels, and their distribution within the operon; *pduB* and *pduC* are more promoter-proximal and are therefore expected to be more highly expressed, whereas *pduO* is relatively promoter-distal and is therefore expected to be expressed at relatively lower levels. Indeed the proteomic data for each of these proteins seemed to support these predictions. Immunoblots of protein samples derived from cells grown under each condition confirmed that these Pdu proteins were essentially absent from the log and stationary phase cells but were present in cells grown in MgM (Fig. 4). During both log phase and MgM conditions, PduO was detected at somewhat lower levels than either PduB or PduC, corroborating the ability of proteomics to discern relative protein levels. Additionally we observed the two forms of PduB (-B and -B′) that have been described previously (45). Although our data clearly demonstrate that PduB and PduB′ share a common C terminus, it remains to be shown whether these are the products of two overlapping genes or whether this shared C terminus represents the post-translational processing of a single gene product.

The immunodetection of the Pdu proteins primarily in the

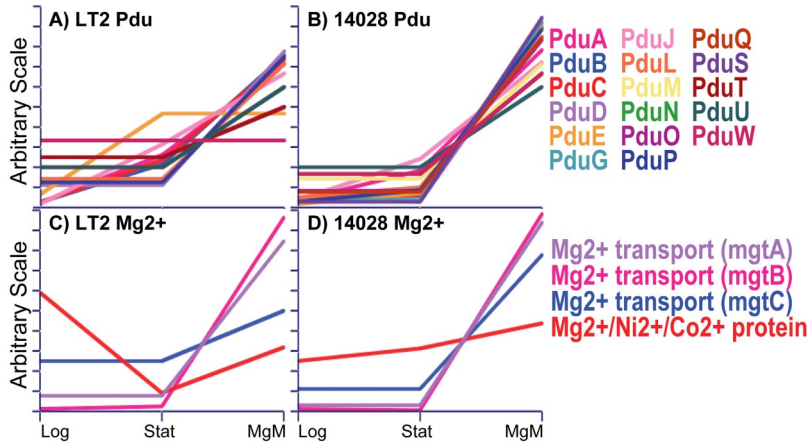

FIG. 3. **Propanediol utilization and magnesium transport proteins are relatively abundant in MgM condition in either strain.** A graphic representative of observation counts for a subset of proteins observed in both 14028 and LT2 strains is shown. The *horizontal axis* represents the three conditions compared, and the *vertical axis* is the normalized observation counts on a scale of 0 to 1. *A* shows the Pdu subset of proteins observed in each condition for the LT2 strain, whereas *B* is for the 14028 strain. *C* shows the Mg^{2+} transport protein subset observed in each condition for the LT2 strain, whereas *D* is for the 14028 strain.

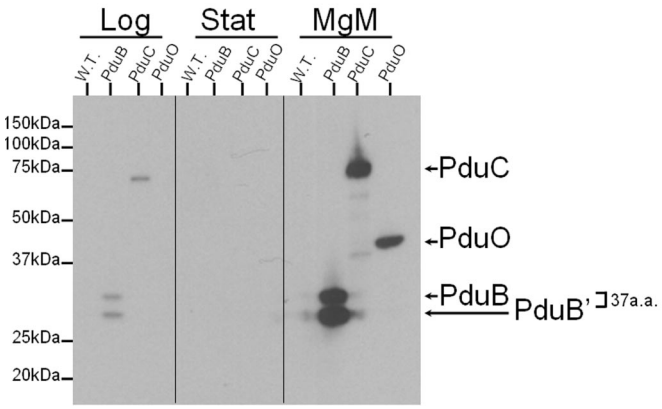

FIG. 4. **Immunoblot analysis of PduB/C/O protein levels from *S. typhimurium* log phase LB, stationary phase LB, and MgM cultures.** Strains expressing HA-tagged variants of each of these *pdu* genes were cultured as described under "Materials and Methods," and protein samples corresponding to equivalent cell numbers were probed for the presence of each epitope-tagged protein. No Pdu proteins were detected during stationary phase in rich medium, whereas PduB (and PduB′, a naturally occurring version missing the N terminus) and PduC were only present at low levels during log phase growth. Exposure to MgM strongly induced the synthesis of all four proteins. *W.T.*, wild type; *Stat*, stationary; *a.a.*, amino acids.

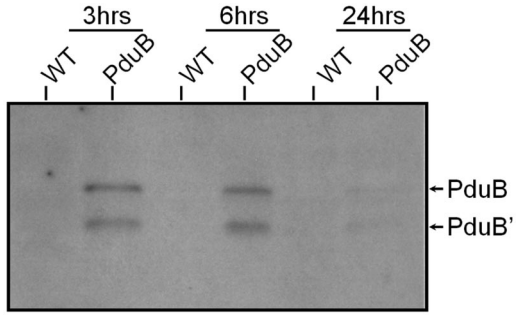

FIG. 5. **Immunoblot analysis of PduB protein levels from *S. typhimurium* during a time course analysis an infection of a RAWS mouse macrophage cell line.** The wild-type (*WT*) control strain and the PduB HA-tagged variant strain were used to demonstrate that the PduB protein appears to be part of an early response in the adaptation to the macrophage lifestyle.

MgM culture condition represented validation of the proteomic observations. To evaluate the presence of these proteins in a more relevant environment for infection, the PduB mutant was used with immunodetection to probe expression in macrophages with time and by cellular compartment. The time course experiment suggested that PduB is expressed in the early phase of macrophage infections, but the concentration is reduced by 24 h (Fig. 5). This exciting observation matches ongoing experiments in model culture conditions suggesting that Pdu proteins are present in early and absent in late macrophage-like culture conditions.[3] Next two different macrophage cell lines were tested for evidence of PduB presence and then probed for possible secretion into the host macrophages. This result demonstrated that PduB did grow successfully in both macrophage cell lines and that the protein was isolated to the *Salmonella*-containing fraction rather than the macrophage cytosol (Fig. 6). These observations taken together demonstrate the utility of the model MgM culture conditions and help highlight a possible role for the Pdu proteins in macrophage infections in mice.

DISCUSSION

This research constitutes the first of a multipart effort to make proteomic results available for the development of vaccines and other therapeutic agents specific to *S. typhimurium*. Because these results will be archived and compared across large numbers of subsequent studies (www.proteomicsresource.org/), a standardized method of sample preparation is required. After evaluating four methods in terms of comprehensive proteomic coverage with minimal sample manipulation, Rapigest appeared to provide the best performance because of its fast and simple protocol as well as its high degree of overlap of protein identifications with the other methods. Because the insoluble protein preparation proved to be more specific to

[3] J. Gustin, J. Adkins, and F. Heffron, unpublished observation.

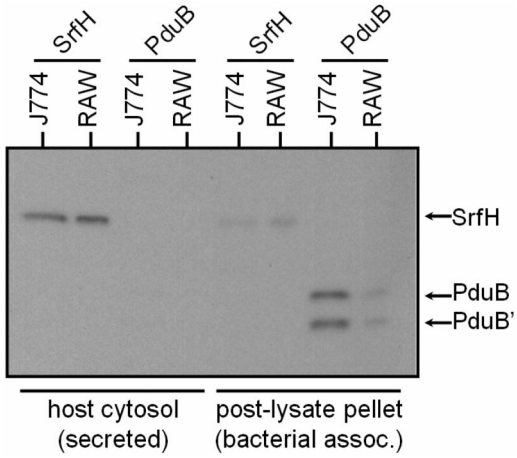

FIG. 6. **Immunoblot analysis of PduB and SrfH protein levels and localization from *S. typhimurium* during an infection of two mouse macrophage cell lines, J774 and RAWS.** A mutant strain with a protein, SrfH, known to be secreted into the macrophage cytosol was generated in the same manner as the PduB HA variant strain. This protein represented a control for function in macrophage and was contrasted with the PduB protein for approximate localization. PduB showed growth in both macrophage cell lines, demonstrating the utility of the MgM culture condition as a model of infection. Further it can be observed that the PduB protein is not secreted into the cytosol of the host macrophage at least not as part of a soluble protein complex. *assoc.*, association.

membrane proteins, a combination of the two methods would increase proteome coverage in future analyses.

This global proteome analysis offers the deepest proteome coverage of *S. typhimurium* to date and compares very well with an important earlier proteome analysis of this organism (46). In fact, we detected 340 proteins (with a minimum of nine peptide observations per protein) of the 350 proteins reported by the previous study. Of the 10 proteins not detected, three were not present in the recent annotation of *S. typhimurium* genome, and at least five of the remaining seven (DcuB, GlpC, TdcB, TdcC, TdcD, YegW, and STM3650) were either anaerobically inducible or labeled solely as putative. Considering our cultures were grown in the presence of oxygen, it is reasonable that gene products whose expression requires anaerobic conditions would not be detected. We believe that the excellent overlap between our effort and the earlier study highlights the quality of both proteome analyses.

Among the most striking biological observations from this study was that the Pdu group of proteins appeared almost exclusively in the MgM growth condition and more often in the 14028 strain. The products of the *pdu* operon allow *S. typhimurium* to grow using propanediol as a sole carbon source that occurs in an adenosylcobalamin-dependent manner. Propanediol is derived from the breakdown of rhamnose and fucose, which one would not expect in a macrophage-phagosome (47). Curiously in addition to the enzymes needed for the catabolism of propanediol, the *pdu* operon has also been shown to encode the structural components of a large proteinaceous microcompartment (related to carboxysomes) whose function remains unclear (45, 48). Several genes in both the propanediol and ethanolamine clusters (that encode the adenosylcobalamin-dependent catabolism of ethanolamine) have been identified in previous studies as having a role in pathogenicity (26, 49–51). The *pdu* operon has also been shown to be regulated by CsrA and Fis, which are both regulators of *Salmonella* pathogenicity (52, 53). In addition, a recent publication that describes a unique "literature mining" approach to finding overlooked connections between microbial genes and phenotypes suggests that there is a correlation between the ability to cause food poisoning and the presence of *pdu* genes (50). These observations, coupled with our own, suggest that a more thorough investigation of the role of the *pdu* operon in *S. typhimurium* pathogenesis is warranted.

Proteome comparisons of various *S. typhimurium* strains cultured under a variety of conditions represent a significant opportunity for the *Salmonella* and proteomic research communities because the results are being made publicly available and queryable, *e.g.* to obtain a qualitative sense of the relative protein abundance. For example, an interesting observation made by Kelly *et al.* (52) was that Fis, a global transcriptional regulator that controls both metabolism and type III secretion systems in *S. typhimurium*, appeared to be abundant in log phase and nearly absent in stationary phase, consistent with our results. The opportunity now exists to cross-validate their observations by simply querying our database and further extend the observations to MgM. Similarly the database can be used to compare gene expressions in both LT2 and 14028. Future efforts may supplement these results across new growth conditions, *e.g.* anaerobic, to further increase the utility of these data.

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‖ To whom correspondence should be addressed: Pacific Northwest National Laboratory, P. O. Box 999, MSIN: K8-98, Richland, WA 99352. E-mail: rds@pnl.gov.

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