THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 39, pp. 29131–29140, September 29, 2006
Printed in the U.S.A.

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[*] [S]

Received for publication, May 15, 2006 Published, JBC Papers in Press, August 7, 2006, DOI 10.1074/jbc.M604640200

Liang Shi[‡1], Joshua N. Adkins[‡], James R. Coleman[‡], Athena A. Schepmoes[‡], Alice Dohnkova[‡], Heather M. Mottaz[‡], Angela D. Norbeck[‡], Samuel O. Purvine[‡], Nathan P. Manes[‡], Heather S. Smallwood[‡], Haixing Wang[‡], John Forbes[§], Philippe Gros[§], Sergio Uzzau[¶], Karin D. Rodland[‡], Fred Heffron[‖], Richard D. Smith[‡], and Thomas C. Squier[‡]

From the [‡]Pacific Northwest National Laboratory, Richland, Washington 99354, [§]McGill University, Montreal, Quebec H3G 1Y6, Canada, [¶]University of Sassari, 07100 Sassari, Italy and [‖]Oregon Health Sciences University, Portland, Oregon 97201

To evade host resistance mechanisms, *Salmonella enterica* serovar Typhimurium (STM), a facultative intracellular pathogen, must alter its proteome following macrophage infection. To identify new colonization and virulence factors that mediate STM pathogenesis, we have isolated STM cells from RAW 264.7 macrophages at various time points following infection and used a liquid chromatography-mass spectrometry-based proteomic approach to detect the changes in STM protein abundance. Because host resistance to STM infection is strongly modulated by the expression of a functional host-resistant regulator, *i.e.* natural resistance-associated macrophage protein 1 (Nramp1, also called Slc11a1), we have also examined the effects of Nramp1 activity on the changes of STM protein abundances. A total of 315 STM proteins have been identified from isolated STM cells, which are largely housekeeping proteins whose abundances remain relatively constant during the time course of infection. However, 39 STM proteins are strongly induced after infection, suggesting their involvement in modulating colonization and infection. Of the 39 induced proteins, 6 proteins are specifically modulated by Nramp1 activity, including STM3117, as well as STM3118–3119 whose time-dependent abundance changes were confirmed using Western blot analysis. Deletion of the gene encoding STM3117 resulted in a dramatic reduction in the ability of STM to colonize wild-type RAW 264.7 macrophages, demonstrating a critical involvement of STM3117 in promoting the replication of STM inside macrophages. The predicted function common for STM3117–3119 is biosynthesis and modification of the peptidoglycan layer of the STM cell wall.

As a facultative intracellular pathogen, *Salmonella enterica* serovar Typhimurium (STM)[2] is the causative agent of gastroenteritis in humans and a lethal systemic infection in BALB/c mice that lack a functional host-resistant factor, natural resistance-associated macrophage protein 1 (Nramp1, also known as Slc11a1). Because its symptoms resemble human typhoid fever caused by the *S. enterica* serovar Typhi, STM-mediated systemic infection in BALB/c mice represents an established model system to investigate the pathogenesis and immunology of typhoid fever in humans (1).

In BALB/c mice, STM invades mainly microfold (M) cells in Peyer's patches after oral ingestion and is then taken up by the phagocytes adjacent to the infected M cells. From there, STM disseminates though the lymphatic system and replicates within the macrophages of spleen and liver (2, 3). Macrophages play a dual role in STM-mediated systemic infection. In naive mice, macrophages are directly involved in controlling the morbidity and mortality of infected mice. Macrophages also function as immune effectors to help in the clearance of STM and recovery from infection in vaccinated mice (4). Thus, whether STM is able to successfully establish a systemic infection in mice depends on its ability to colonize and proliferate inside host macrophages. Indeed, STM strains unable to replicate inside macrophages fail to cause systemic infection and are avirulent (5).

After uptake by Nramp1-negative (N−) macrophages, STM resides in a unique cellular compartment called the *Salmonella*-containing vacuole (SCV). Unlike normal phagosomes, most SCVs become segregated from late endosomes and lysosomes and lack lysosomal hydrolases. In addition, the fusion is blocked between most SCVs with newly formed vesicles containing functional phagocyte NADPH oxidase or inducible nitric-oxide synthase, which generate antibacterial reactive oxygen and

* This work was supported in part by the Laboratory Directed Research and Development Program of the Department of Energy (to L. S.) and by NIAID, National Institutes of Health/Department of Health and Human Services Interagency Agreement Y1-AI-4894-01. Significant portions of this work were carried out at the W. R. Wiley Environmental Molecular Sciences Laboratory, a national scientific user facility sponsored by the Department of Energy Office of Biological and Environmental Sciences program located at Pacific Northwest National Laboratory. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1 and Tables S1–S3.

[1] To whom correspondence should be addressed: Microbiology Group, Pacific Northwest National Laboratory, 902 Battelle Blvd., P. O. Box 999, MSIN: P7–50, Richland, WA 99354. Tel.: 509-376-4834; Fax: 509-372-1632; E-mail: liang.shi@pnl.gov.

[2] The abbreviations used are: STM, *S. enterica* serovar Typhimurium; AMT, accurate mass and time; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline; FTICR, Fourier transform ion cyclotron resonance; HBSS, Hanks' buffered saline solution; IHF, integration host factor; LC, liquid chromatography; MG, methylglyoxal; MS, mass spectrometry; Nramp1, natural resistance-associated macrophage protein 1; N+, Nramp1-positive; N−, Nramp1-negative; p.i., post infection; SCV, *Salmonella*-containing vacuole; SPI, *Salmonella* pathogenicity island; T3S, type III secretion system; WB, Western blot; NET, normalized elution time; WT, wild type.

nitrogen species, respectively. One of the STM virulence systems directly involved in regulating the biogenesis of SCV is the type III secretion system (T3S) encoded by *Salmonella* Pathogenicity Island-2 (SPI-2). Found in many Gram-negative pathogenic bacteria, T3S is a needle-like structure that physically connects the cytoplasms of bacterial and host cells, permitting direct translocation of bacterial effector proteins into the cytosol of host cells to alter the function of host cells in favor of disease progression (6–9). Nramp1 activity competes with the ability of STM to control the biogenesis of SCV, probably via up-regulation of some SPI-2-related genes, and increases the traffic of lysosomal hydrolases to the SCV to facilitate the killing of STM (10, 11).

In addition to T3S of SPI-2, large numbers of other STM proteins, such as the proteins directly regulated by the PhoP/PhoQ two-component system, are also involved in promoting the colonization of STM within macrophages in response to stressors, such as nutritional limitations and the damage done by antibacterial peptides, that STM encounters in the SCV (12–14). Almost all of these proteins have been identified using traditional molecular, genetic, and/or biochemical means, and their identification has helped greatly to unravel the molecular mechanisms underlying the ability of STM to survive inside macrophages.

Different mass spectrometry (MS)-based methods have been used to identify STM proteins. After separation by two-dimensional electrophoresis, ~233 STM proteins were identified from STM cultured under varied growth conditions (15–17). Using two-dimensional chromatography with on-line mass detection, Coldham and Woodward (18) detected 816 individual STM proteins from the STM harvested from culture medium. From STM grown under three different culture conditions, Adkins *et al.* (19) identified a total of 2,343 STM proteins with a liquid chromatography (LC)-MS-based "bottom-up" proteomic approach. Recently, 898 STM proteins were detected in the samples isolated from the spleens and/or caecum of infected N− mice (20). However, no measurements have been reported in which time-dependent changes in the abundance of STM proteins are determined following the infection of macrophages.

To identify key proteins linked to the successful colonization of macrophages, we used an accurate mass and time (AMT) tag proteomic approach developed at Pacific Northwest National Laboratory to identify the STM proteins isolated from the RAW 264.7 macrophages with or without functional Nramp1 at different time points of infection. These results represent the first systematic investigation of changes in the abundance of bacterial proteins associated with macrophage colonization by using a global proteomics approach. Systematic investigation of the bacterial proteins important for colonization of macrophages will help to identify the potential candidates that can be used in the detection of bacterial pathogens and the development of effective vaccines.

EXPERIMENTAL PROCEDURES

Reagents and Standard Procedures—All cell culture reagents were purchased from Invitrogen. Protein concentrations were measured with a BCA protein assay kit from Pierce. A bacterial DnaK antibody was purchased from StressGen (Victoria, British Columbia, Canada). All other antibodies and interferon-γ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). SDS-PAGE and Western blot (WB) were conducted according to the instructions from Invitrogen. All chemicals used for tryptic digest were purchased from Sigma unless otherwise noted.

TABLE 1
Bacterial strains and plasmids used in this study

	Relevant genotype	Source or Ref.
Strains		
STM14028	Wild type	ATCC
LS1001[a]	+ pKD46	This study
LS1026	ΔSTM3117	This study
LS1028	STM4319 + 3 × FLAG[b]	This study
LS1029	STM3117 + 3 × FLAG	This study
LS1052	STM2777 + 3 × FLAG	This study
LS1054	STM3118 + 3 × FLAG	This study
LS1059	STM3119 + 3 × FLAG	This study
Plasmids		
pKD46	λ Red (γ, β, and *exo*)	30
pKD4	kan^R	30
pCP20	FLP^+	57
pSUB11	Coding sequence for 3 × FLAG	31

[a] All STM strains were generated from wild-type STM14028.
[b] The sequence used is as follows: DYKDHDGDYKDHDIDYKDDDDK.

Bacterial Strains and Culture Conditions—Bacterial strains and plasmids used in this study are listed in Table 1. All STM strains were grown in Luria-Bertani (LB) medium. Kanamycin and ampicillin were used at 50 μg/ml.

Cell Culture and Infection—The cell culture conditions for maintaining RAW 264.7 macrophage-like cell line that expresses functional Nramp1 (Nramp1-positive or N+) and its parental wild-type (WT) RAW 264.7 cell line deficient in a functional Nramp1 (Nramp1-negative or N−) were described previously (21). One day before infection, cells were harvested, counted with a Coulter Z^{TM} Series cell counter (Beckman Coulter, Fullerton, CA), and seeded at 1×10^6 cells/well in 6-well tissue culture plates. After the cells adhered to the bottom of the wells, they were treated with 2 ml/well of recombinant interferon-γ (100 units/ml) for 21–24 h. Cultures of STM were prepared from frozen stocks in LB and grown for 18 h at 37 °C. They were harvested, washed once with the same volume of Dulbecco's phosphate-buffered-saline (DPBS), and resuspended in 1 ml of DPBS. After their concentrations were determined, STM cells were diluted in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and incubated on ice for 30 min. After macrophage cells were washed twice with 2 ml/well of Hanks' buffered saline solution (HBSS), 1 ml of the STM in DMEM was added in each well with a multiplicity of infection of 100. To increase the uptake of STM, plates were centrifuged at 1000 × *g* for 10 min. Uptake of STM was allowed to occur for 30 min at 37 °C in 5% CO_2. This time point was defined as 0 h post-infection (p.i.). After cells were washed three times with 2 ml/well of HBSS to remove the STM that were not taken up by the cells, 2 ml of DMEM supplemented with fetal bovine serum and 12.5 μg/ml of gentamicin was added to each well.

Isolation of STM Cells—At different predetermined time points, the cells were washed twice with 2 ml/well of HBSS and then used for isolating STM cells. Two different methods were

used to isolate STM cells, and all procedures were carried out at 4 °C. Method 1 requires ~4 h, does not involve the use of any detergents, and was originally developed as a gentle procedure to isolate organelles, including the phagosome/SCV. This procedure employed 1 ml/well of homogenization buffer (20 mM HEPES-KOH, pH 7.2, 250 mM sucrose, and 0.5 mM EGTA) to lift the cells (22). The cells from two plates were pooled and lysed by passing cells 30 times through a ball bearing homogenizer with a clearance of 12 μm (Isobiotech, Heidelberg, Germany). Homogenates were then centrifuged at 400 × *g* for 5 min. The supernatants were frozen in liquid nitrogen, thawed at room temperature, diluted with 3 volumes of homogenization buffer, and centrifuged at 12,000 × *g* for 1 min. The pellets were resuspended in homogenization buffer, loaded on 10 volumes of 12% sucrose cushion, and centrifuged at 1700 rpm for 45 min. The STM cells were recovered from the bottom of the centrifuge tubes with 100 μl of 100 mM NH_4HCO_3, pH 8.4.

In contrast, method 2 involves the differential solubilization of the host membranes by taking advantage of the detergent resistance of the outer membrane of STM cells in comparison to the membranes from macrophages (23). After washing with HBSS, cells were lysed for 30 min with 1 ml/well of cell lysis solution (0.1% (w/v) SDS, 1% (v/v) acidic phenol, and 19% (v/v) ethanol in double distilled water). The cell lysates from two plates were pooled and centrifuged at 5,000 × *g* for 20 min. The pellets were washed twice with HBSS and resuspended in 100 μl of 100 mM NH_4HCO_3, pH 8.4. The isolated STM cells were analyzed subsequently by electron microscopy, LC-MS or WB.

Electron Microscopy—Portions (50 μl) of isolated STM cells were diluted with 1 ml of 0.1 M cacodylate buffer, pH 7.2, washed twice with the same buffer, and fixed with 1% glutaraldehyde in 0.1 M cacodylate buffer at 4 °C for 2 h. Cells were then washed three times in cacodylate buffer and exposed to 1% OsO_4 for 1 h. After three additional cacodylate washes, cells were gradually dehydrated using a series of ethanol solutions, including three washes in 100% ethanol, 10 min each. Samples were infiltrated with 50:50% ethanol and LR White resin (Electron Microscopy Sciences, Hatfield, PA) and washed three times in 100% LR White, 15 min each. The cellular material was transferred to gelatin capsules filled with LR White and polymerized at 60 °C overnight. Hardened blocks were sectioned on an ultramicrotome (Ultracut UCT, Leica, Germany) to a thickness of 70 nm, and sections were mounted on 200 mesh copper grids with Formvar film sputtered with carbon (Electron Microscopy Sciences). Sections were post-stained with 1% uranyl acetate and lead citrate (Electron Microscopy Sciences) to provide contrast. They were imaged using a JEOL 2010 HR transmission electron microscope operating at 200 kV. Images were collected digitally on a CCD camera (Gatan, Pleasanton, CA) by using Digital Micrograph software.

Lysis of STM Cells and Tryptic Digestion—STM cells were lysed by bead beating (maximum speed, 5 min, 20 °C) in a Mini-Bead-Beater-8™ (BioSpec Products, Bartlesville, OK) with 0.1-mm zirconia/silica beads in 2-ml sterile microcentrifuge tubes. The supernatants were collected by centrifugation at 15,000 × *g* for 5 min. After protein concentrations were determined, urea, thiourea, and dithiothreitol were added to the supernatants at final concentrations of 7 M, 2 M, and 5 mM, respectively, and incubated at 60 °C for 30 min. The samples were then diluted 10-fold with 100 mM NH_4HCO_3 in the presence of 1 mM $CaCl_2$ and subjected to digestion by trypsin (Promega, Madison, WI) at 1:50 w/w trypsin-to-protein ratio for 3 h at 37 °C. The resulting peptides were desalted with strong cation exchange columns (Supelco, Bellefonte, PA) and concentrated with a SpeedVac. The resulting peptide concentration was determined using a BCA protein assay kit, and the trypsin-digested peptides were quickly frozen in liquid N_2 and stored at −80 °C until analysis with LC-MS (24).

LC-MS Analysis and Peptide Identification—All samples were analyzed by following protocols established previously (25, 26). Briefly, samples were first analyzed with a ThermoElectron model LTQ ion trap mass spectrometer (ThermoElectron Corp., San Jose, CA) coupled to a high pressure and high efficiency capillary LC separation system based upon a reverse-phase C18 as stationary phase. Upon elution from the capillary column, peptides were ionized by electrospray ionization and directly entered the mass spectrometer. Tandem mass spectra (MS/MS) from collisionally induced dissociation of peptides in the ion trap were used to identify the peptides using the SEQUEST™ program (27). Peptides were considered of acceptable confidence for population into the initial AMT tag data base if the scores provided by SEQUEST passed those filters used by Washburn *et al.* (28) with the additional criteria of a minimum discriminant score (29) of 0.5 (on a 0–1 scale, with 1 being the highest quality match). The data base that contained both the STM LT2 and mouse data bases was used for the searches for peptides, and both STM and mouse peptides were identified and populated into the same AMT tag data base.

In parallel to the building of the initial AMT tag data base, all samples were analyzed with a 9.4-tesla Fourier transform ion cyclotron resonance (FTICR) mass spectrometer (Bruker Daltonic, Billirica, MA) after the peptides were separated by reverse-phase capillary high pressure liquid chromatography under identical conditions as described above. Each biological sample was analyzed in triplicate on the FTICR mass spectrometer. Relevant information such as the elution time from the capillary LC column, the abundance of the signal (integrated area under the elution profile), and the monoisotopic mass (determined from charge state and the high accuracy m/z measurement) of each feature observed in the FTICR was used to match the peptide identifications contained within the initial AMT tag data base. A matching feature was required to be within 5 ppm of the peptide mass and the normalized elution time (NET) within 5%. These peptides, now identified and quantified, were used to infer the protein composition of the samples. All proteins were required to have at least three observed peptides to be included in the confident results. In addition, the number of peptides observed for each protein in a biological sample was divided by the total number of peptides determined from the same sample to give an estimate of relative abundance of each identified protein in the sample. Heat maps were generated using the software tool OmniViz™3.8 (OmniViz, Maynard, MA). The "relative risk" (RR) of each LC-FTICR data set was calculated (RR = (RRL − RRT)/RRL) from the number of matching AMT tags within a tight (±5 ppm and ±3% NET) error window (RRT) to the number matching

TABLE 2
Primers used in this study
The sequences are from 5′ to 3′.

Primer	Sequence
2777TF	ACCGGGGCGGGCTTATTATGCCGGTGTGACGGCCTCGTTTGACTACAAAGACCATGACGG
2777TKR	CCGCCAGAATATGCCCGCCAGCGGCGGGCATAATCCATCACATATGAATATCCTCCTTAG
3117TF	GGACCCTGATGGAAACTTAATTGAAATTTCGCAGTATGTTGACTACAAAGACCATGACGG
3117TKR	GTGATCATAATAAAGGCAGTAATAGTAAGTTTTAACATTACATATGAATATCCTCCTTAG
3117KF	GTAGATGCAAAAAAAATGCATCTGATTTAAAAGTAGGTTAGTGTAGGCTGGAGCTGCTTCG
3118TF	GCAGCAACTTAGAGTAGATGCAAAAAAAATGCATCTGATTGACTACAAAGACCATGACGG
3118TKR	TAGGGATGCTACATTAAAAAATAGCATTAACCTACTTTTACATATGAATATCCTCCTTAG
3119TF	TTATTGCTGTGGATGTTCGCCTGAAGAAGACGCCGCTTATGACTACAAAGACCATGACGG
3119TKR	TAACATTCATCATGACTCTAAACCTCAGGGCGATTTCTCACATATGAATATCCTCCTTAG
4319TF	TAATTTATTGAGTAAAGAAGATCACCCCAAACTTAATTACGACTACAAAGACCATGACGG
4319TKR	CATTTGCTGTGGCCAGTTTGCGGGAAGACTTTCACCTTCACATATGAATATCCTCCTTAG

within a loose (±10 ppm and ±6% NET) error window (RRL). The overall false-positive rate of peptide identification was estimated by averaging the relative risks of all of the FTICR data sets.

Genetic Manipulation of Chromosomal Genes of STM—The primers used for tagging or deleting chromosomal genes of STM were listed in Table 2. The procedures of gene tagging and deleting, which were mediated by phage λ-Red recombinase, were the same as those described previously (30, 31). For gene tagging, the sequences encoding 3 × FLAG epitopes were inserted in-frame at the 3′-end of the coding regions immediately before the stop codons of all targeted STM genes. Gene deletion was carried out to eliminate the entire coding region of the target. After validation with PCR, the antibiotic resistance gene that served as a selective marker was removed to minimize polar effects. All resulting STM strains made in this study are listed in Table 1. The strains tagged with the 3 × FLAG epitope were used to validate the LC-MS results by WB analysis of the expression of each tagged STM proteins isolated from macrophages. The gene-deletion strain was tested to determine whether its ability to survive inside macrophages was attenuated.

Assay for Survival and Replication of STM inside Macrophages—As described above, the RAW macrophages, and WT and gene-deletion strains of STM were prepared 1 day before infection, with a slight modification in which RAW macrophages were seeded at 5×10^5 cells/well in 24-well tissue culture plates. The infections were carried out with a multiplicity of infection of 100. At pre-determined time points, cells were washed twice with 1 ml/well of HBSS and lysed with 0.5 ml/well of cell lysis buffer (1% (v/v) of Triton X-100, 0.1% (w/v) SDS in DPBS) at room temperature for 5 min. The lysis solution in each well was pipeted up and down 10 times and diluted by several orders of magnitude, and each dilution was plated on LB agar plates. After incubation at 37 °C for 16 h, the numbers of colony-forming units on each LB agar plate were counted to determine the numbers of live STM cells in each well, and a minimum of three wells was counted for each measurement (32).

Statistical Analysis—All values are expressed as means ± S.D. Student's *t* test was used for comparing groups.

RESULTS

Isolation of STM Cells from Infected Macrophages—Critical to the identification of functionally important proteins that mediate the colonization of macrophages by *Salmonella* is the enrichment of STM from infected host cells and the retention of STM associated proteins in a form suitable for LC-MS analysis. To accomplish this goal, we have used two complementary methods of STM isolation, which respectively rely on the gentle physical disruption of isolated cells to maintain organelle structures (method 1) and the differential solubilization of the host membranes by taking advantage of the detergent resistance of STM cells (method 2). For both methods, STM were isolated from WT (N−) macrophages at 2 h p.i., and the relative purity of the preparation and abundance of isolated STM proteins was compared using EM and LC-MS, respectively.

EM examination of STM isolated by method 1 revealed large numbers of vesicle-like structures derived from organelles within macrophages that were co-isolated with STM cells (Fig. 1*A*). Consistent with these results, LC-MS analysis found that only 33.9 ± 0.7% of total identified proteins originated from the STM, whereas the remainder were mouse proteins (Fig. 1, *C* and *D*). In contrast, EM analysis of the isolated STM from method 2 indicates a highly purified preparation of STM cells with much fewer macrophage-derived vesicle-like structures (Fig. 1*B*). Indeed, MS analysis confirmed that 67.3 ± 3.4% of the total proteins prepared from this latter method were derived from STM. Furthermore, 74% of the bacterial proteins identified in method 1 were also found in the samples isolated by method 2. Given the much greater purity of the preparation obtained using method 2, and the ability to rapidly compare the time course of infection using this method to prepare multiple samples in parallel, subsequent measurements to investigate time-dependent changes in the abundances of STM proteins following bacterial internalization within macrophages emphasized this preparation.

Modulation of Salmonella Colonization by Nramp1 Activity—Wild-type (N−) RAW 264.7 macrophages are rapidly colonized by STM cells, which remain viable for more than 24 h following their uptake into macrophages (Fig. 2*A*). In contrast, expression of Nramp1 in RAW 264.7 (*i.e.* N+) results in the rapid clearance of STM from macrophages. To identify proteins whose expression facilitates bacterial colonization of macrophages, we have isolated STM cells from both N− and N+ macrophages and have identified both bacterial and mouse proteins using LC-FTICR MS. As already indicated (see above), the majority of identified proteins is derived from the STM at early stages of infection. There is a decrease in the yield of bacterial

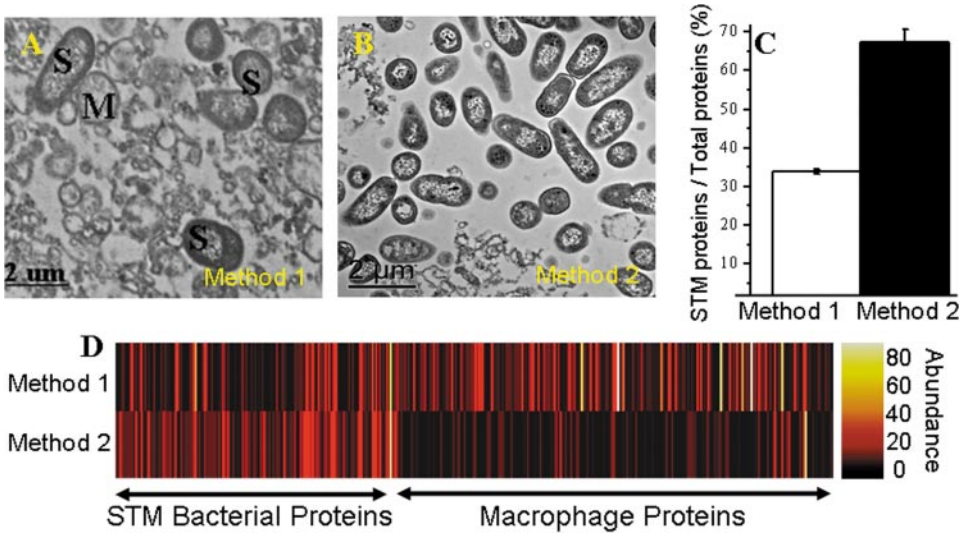

FIGURE 1. **Optimal method for isolation of STM cells.** Electron microscopy showing STM cells (*S*), mitochondrion (*M*) (*A* and *B*), and proteomic characterization (*C* and *D*) of preparations of enriched STM cells isolated from N− RAW 264.7 macrophages at 2 h p.i. following either mild cell disruption and centrifugation (*Method 1*) or the differential solubilization of macrophage (*Method 2*). *D,* heat map representation shows all identified proteins from methods 1 and 2, where colors represent the peptide abundance for each protein ranging from *black* (total number of peptides = 0) to *white* (total number of peptides = 81).

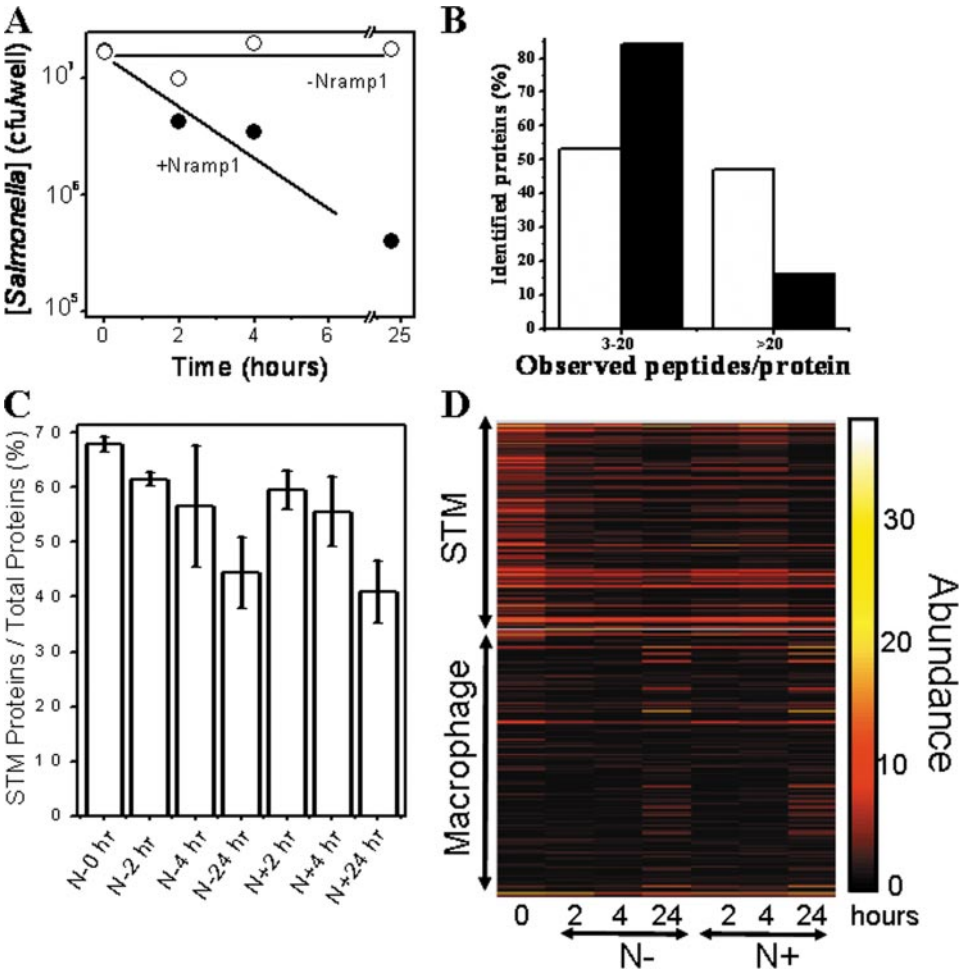

FIGURE 2. **STM abundance changes following infection.** Viability of STM cells (*A*), bacterial protein yields (*C*), and abundance changes of identified proteins (*D*) at indicated times post-infection for N− and N+ 264.7 macrophages. Fraction of STM (*open bars*) and macrophage (*solid bars*) proteins identified from all experiments in which peptide counts were more/less than 20 peptides (*B*). Peptide abundances for individual proteins (*D*) at each time point, where colors range from *black* (total number of peptides = 0) to *white* (total number of peptides = 39).

proteins relative to those derived from the macrophage host at longer times following infection (Fig. 2*C*). However, even after 24 h p.i. in N+ macrophages, where greater than 95% of internalized bacteria have been killed, over 40% of the identified proteins are of bacterial origin. The preponderance of bacterial proteins identified is consistent with a high level of STM purification (Fig. 1*B*). Our observation of gradually increased numbers of mouse proteins detected among the samples isolated from 0 to 24 h p.i. is also consistent with previous results that SCV progressively interacts with other macrophage proteins during the course of SCV biogenesis (8).

A total of 16,245 peptides were identified from all samples isolated from both N− and/or N+ RAW 264.7 macrophages (Table 3). The false-positive rate of peptide identification throughout this study was determined to be 4 ± 1%. Among the identified peptides, 10,649 (66%) are derived from 315 STM proteins and 5,596 peptides (34%) were derived from 371 mouse proteins (Table 3, also see supplemental Table S1). Consistent with our proteomic data, message RNA levels associated with 296 of the 315 identified STM proteins (94%) have been identified previously for STM isolated from N− macrophages (23). Large peptide counts (*i.e.* >20 peptides/protein) were observed for approximately one-half of the bacterial proteins (Fig. 2*B*). In contrast, only 16% of the macrophage host proteins were observed to have similar peptide abundances, consistent with the large enrichment of STM in the isolated preparation (Fig. 1*B*). Thus, the predominance of bacterial proteins in this preparation of STM isolated at various times p.i., coupled with the sensitivity and large dynamic range of the LC-FTICR instrument, permits a sensitive identification of protein abundance changes during the time course of STM colonization within macrophages.

From the peptide abundances, it is apparent that the majority of the bacterial proteins do not change following infection, although signifi-

TABLE 3
The numbers of peptides and proteins identified

	STM	Mouse	Total	STM/total
				%
Peptides	10,649	5,596	16,245	65.6
Proteins	315	371	686	45.9

cant decreases in the relative abundance of selected proteins is apparent following the initial colonization of macrophages (Fig. 2*D*). Consistent with this result, the physiological functions of most of the identified STM as well as macrophage-derived proteins are related to housekeeping functions, involving the biosynthesis and metabolism of amino acids, carbohydrate, lipids, proteins, and nucleotides, energy production, and cellular process (supplemental Fig. S1). Nine STM proteins related to SPI-1, SPI-2, and SPI-3 were identified to change during the time course of infection (supplemental Table S1, and also see below). The physiological functions of 11% of STM proteins identified remain unknown or unclassified. In contrast, close to 27% of identified mouse proteins had no assigned functions.

Quantitative Agreement between MS-derived Peptide Abundances and Protein Immunoreactivities—STM DnaK (STM0012) and mouse α-tubulin (gi 6755901) are abundant proteins that are present in every sample analyzed by LC-MS (supplemental Table S2), and the availability of commercial antibodies permits their quantification using WB. In agreement with MS results, immunoblots indicate that there is a large abundance of DnaK, whose expression is relatively insensitive to STM infection (Fig. 3). Likewise, both peptide counts from LC-MS analysis and WB indicate that there is a dramatic increase in the protein abundance of α-tubulin at 24 h p.i. This latter result is consistent with the structural role associated with α-tubulin that in association with β-tubulin forms microtubules that accumulate around SCV in both STM-infected epithelial cells and macrophages at long times (*i.e.* >7 h) p.i. (33). Our detection of increased amounts of α-tubulin that co-purifies with STM at 24 h p.i. supports the hypothesis that observed increases in the relative amounts of mouse proteins detected in STM preparations occur, at least in part, because of specific associations between SCV and macrophage proteins (*e.g.* microtubules) during the course of SCV biogenesis.

Increased STM Protein Abundances during STM Infection—Identification of the STM proteins up-regulated after infecting macrophages will help with understanding the molecular mechanisms that control STM-macrophage interactions as well as the identification of novel virulence factors. To identify these STM proteins, we compared the STM proteins found by LC-MS analysis in the samples isolated at 0 h p.i. to those at 2, 4, and/or 24 h p.i. because STM proteins unique to the samples isolated at 2, 4, and/or 24 h p.i. were most likely up-regulated after infecting macrophages.

Among the STM proteins listed in supplemental Table S1, 39 of them (12%) were found by LC-MS analysis only in the samples isolated at 2, 4, and/or 24 h p.i. (Fig. 4*A*; supplemental Table S3). Consistent with these observations, prior gene expression profiles obtained from STM isolated from N− macrophages have shown that expression of the genes for 17 of these STM proteins (43%) are up-regulated upon STM infection of macrophages (23). Among the 39 identified proteins, 7 are known *Salmonella* virulence factors, which include IHFα (STM1339), IHFβ (STM0982), MgtB (STM3763), OmpR (STM3502), SitA (STM2861), SitB (STM2862), and SodCI (STM1044) (34–39). Furthermore, SerA (STM3062), IHFα, and IHFβ are detected only from N+ macrophages, in agreement with prior gene expression measurements that indicate that expression levels for the genes encoding these three proteins are down-regulated in the STM isolated from N− macrophages (23).

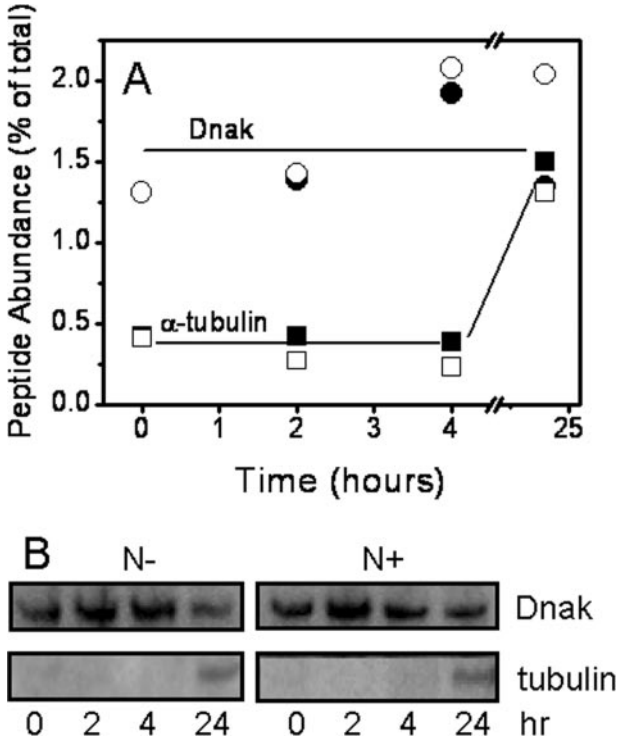

FIGURE 3. **Validation of MS results by Western blot analysis.** Peptide abundances (*A*) and immunoblots (*B*) for STM DnaK (○, ●) or mouse α-tubulin (□, ■) isolated from either N− (●, ■) or N+ (○, □) macrophages. *B*, 5 μg of total protein was separated by SDS-PAGE and probed using specific antibodies, as fully described under "Experimental Procedures."

Confirmation of the up-regulation of IroN (STM2777), PhoN (STM4319), and STM3117 following STM infection of macrophages involved their tagging with a 3 × FLAG epitope. IroN, a TonB-dependent siderophore receptor protein, was investigated to clarify observed protein abundance increases from prior gene expression profiles that indicate this gene to be down-regulated after infection (23). STM3117 lacks a known function and in conjunction with STM3118–3119 has been speculated as a potential virulence factor (23). Because STM3117–3119 are all up-regulated following STM infection of macrophages (Fig. 4*A*) and probably belong to a common operon, an understanding of their regulation may facilitate an appreciation of their function. Finally, PhoN, a nonspecific acid phosphatase, served as a positive control because its gene expression, which is controlled by the PhoP/PhoQ two-component system, is known to be increased upon infection of macrophages (40, 41). WB analyses demonstrate that the abundance of all three tagged proteins increases following STM entry into macrophages. Increases in the abundances of STM3117 and PhoN were evident at 2 h p.i. and peaked at 4 h

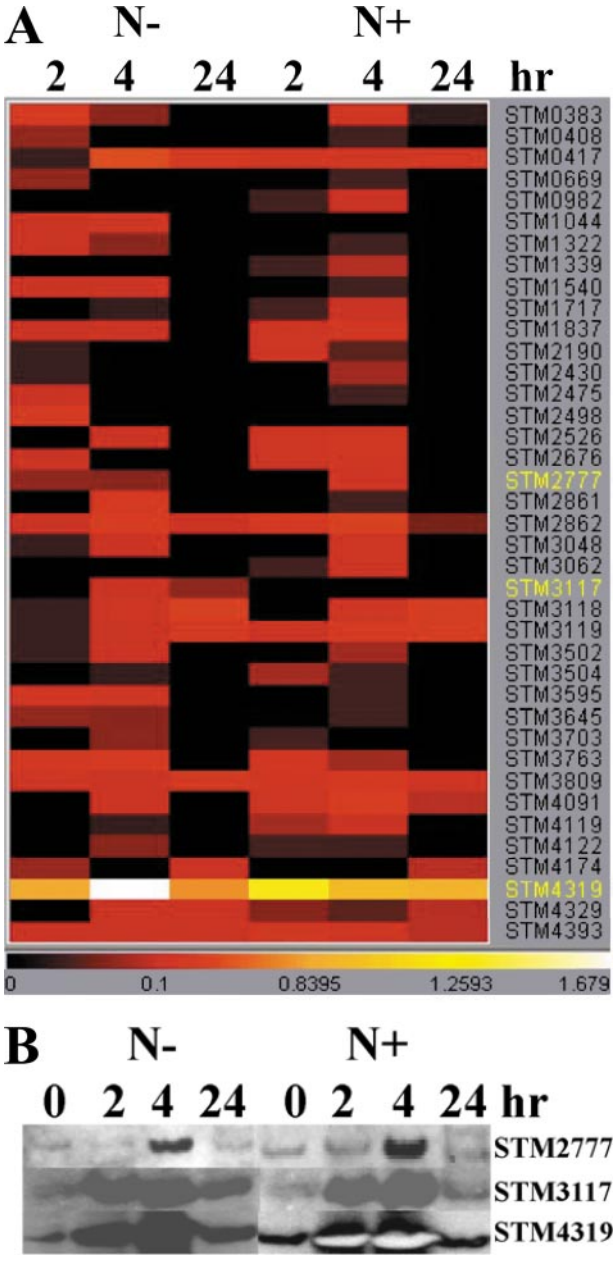

FIGURE 4. **Induced STM proteins following infection of macrophages.** Peptide abundances (*A*) and Western blot analysis against engineered 3 × FLAG epitopes (*B*) depicting changes in protein abundances for indicated protein names following STM infection of N− (*left*) or N+ (*right*) macrophages. Average abundances of peptides detected for each protein were normalized to the total number of identified peptides and varied from *black* (abundance of peptides = 0%) to *white* (abundance of peptides = 1.7%). For Western blot analysis, a total of 10 μg of total protein was separated by SDS-PAGE and probed using anti-FLAG antibodies.

p.i., whereas the increase of IroN could only be detected at 4 h p.i. (Fig. 4*B*). Thus, the LC-MS and WB analyses in this study generally agree very well.

Up-regulation of IroN as well as SitA/B, components of a Mn^{2+} transporter system (42), and MgtB, an Mg^{2+} transporter (43), after infecting macrophages indicates that STM encounters a divalent metal ion-limited condition inside SCV. Furthermore, observed increases in the protein abundances of PhoN and MgtB are consistent with prior measurements that indicate the genes encoding these proteins are regulated by the PhoP/PhoQ two-component system, which is known to be activated following STM infection of macrophages (1, 40). Likewise, our observation that the abundance of OmpR increases after STM infection of macrophages is consistent with previous results indicating that T3S of SPI-2 is up-regulated after STM entry into macrophages (6–9). This observation follows from the fact that one of the major functions of OmpR is to regulate the expression of SsrA/B, which in turn regulates the expression of T3S of SPI-2 (37). However, no other SPI-2 related proteins were detected, consistent with the low abundance of these proteins (*i.e.* STM cells synthesizes on average one T3S structure of SPI-2 (44)) and their mechanisms of action, which involve the translocation of effector proteins of T3S from SPI-2 into the cytoplasm of host cells. Thus, in the process of isolating STM, we would expect to lose many of these proteins.

Nramp1-mediated Changes in STM Protein Abundances—Host resistance is increased upon expression of the divalent metal transporter Nramp1, which is rapidly recruited to the SCV after phagocytosis of STM (45, 46) (Fig. 2*A*). Nramp1 is known to induce the differential regulation of STM genes, which are thought to represent an adaptive response (11). When analyzing STM proteins up-regulated after infection of macrophages, we found that six STM proteins could be detected by LC-MS only in the samples isolated from either N− or N+ macrophages (Fig. 5*A*). IHFα, IHFβ, and SerA are observed only when STM are isolated from N+ macrophages, whereas SodCI, Upp (STM2498), and STM3117 are only observed when STM are isolated from N− macrophages (also see supplemental Table S3).

Three of the identified proteins, *i.e.* IHFα, IHFβ, and SodC, have been shown previously to be involved in the development of STM virulence. IHFα and IHFβ form a heterodimer called integration host factor (IHF). Together, these proteins function as a DNA-binding protein. IHF regulates the transcription of a broad spectrum of STM genes, including those of SPI-1 through SPI-5 as well as *Salmonella* plasmid virulence genes (34, 35). SodCI is one of the two periplasmic (Cu,Zn) superoxide dismutases of STM, which functions to limit the oxidative burst associated with NADPH oxidase in the SCV. Indeed, inactivation of *sodCI* impairs the ability of STM to proliferate in mouse spleen (39). Consistent with our observations, SodCI was found previously in STM-infected N− macrophages and epithelial cells as well as the spleens of mice (39). The absence of SodCI in the STM isolated from N+ macrophages suggests that the Nramp1 activity may prevent the efficient insertion of the Cu/Zn co-factor into SodCI to render STM more susceptible to host-mediated killing. Uracil phosphoribosyltransferase (Upp) functions to regulate entry into the pyrimidine salvage pathway commonly induced by starvation conditions, whereas SerA (D-3-phosphoglycerate dehydrogenase) regulates the cellular levels of 3-phosphoglycerate from serine. Neither has an apparent role in STM pathogenesis (47, 48).

Likewise, STM3117 is observed by LC-MS only in the STM isolated from N− macrophages, suggesting a much greater abundance than present in N+ macrophages, where STM cells are unable to colonize their host (Fig. 5*A*). A detailed analysis of STM3117 tagged with 3 × FLAG by WB confirms that the abundance of STM3117 is substantially higher in N− cells (Fig.

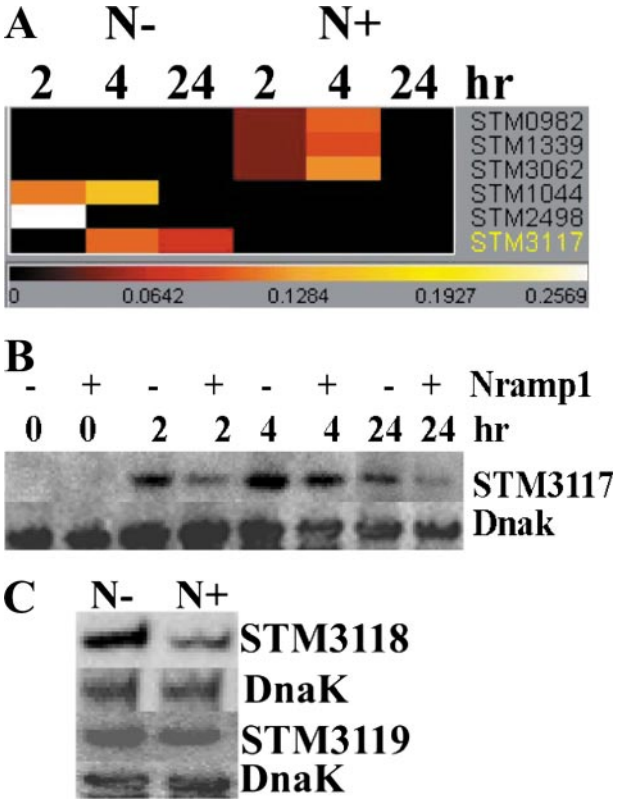

FIGURE 5. **STM proteins affected by Nramp1 expression.** Peptide abundances (*A*) and Western blot analysis (*B* and *C*) for STM proteins that are differentially expressed following STM infection of N− or N+ macrophages. From LC-MS data (*A*), the average abundance of identified peptides for each individual protein is normalized to the total number of identified peptides and varies from *black* (relative abundance peptides = 0%) to *white* (relative abundance of peptide = 0.26%). Western blot analyses illustrate time-dependent changes in the relative abundance of STM3117 in comparison to DnaK (*B*) as well as of STM3118 and STM3119 following 2 h of post-infection for N− or N+ macrophages (*C*). For each lane, 5 μg of total protein was separated by SDS-PAGE and was first probed with an anti-FLAG antibody and then stripped and re-probed using an anti-DnaK antibody.

5*B*). Similar changes in protein abundance are observed for STM3118 and STM3119 in response to Nramp1 expression (Fig. 5*C*), consistent with the genetic organization of the gene cluster encoding these three proteins which suggests they belong to an operon.

Diminished Bacterial Viability upon Deletion of the Gene Encoding STM3117 in N− Macrophages—In an effort to understand its role in mediating the survival of STM inside macrophages, we have deleted the gene encoding STM3117 using the λ-phage recombinase system (30) (Fig. 6*A*). Elimination of STM3117 had no effects on the ability of STM to invade macrophages, as similar numbers of WT and ΔSTM3117 mutant cells were observed following their culture from lysates of N− and N+ macrophages at 0 h p.i. (data not shown). However, deletion of STM3117 results in a dramatic reduction in the replication of STM in N− macrophages (Fig. 6*B*), suggesting that STM3117 contributes to the establishment of bacterial colonization within macrophages. In N+ macrophages STM cells are rapidly killed irrespective of the presence of STM3117, and the ΔSTM3117 mutation has essentially no effect on the time-dependent clearance of the bacteria.

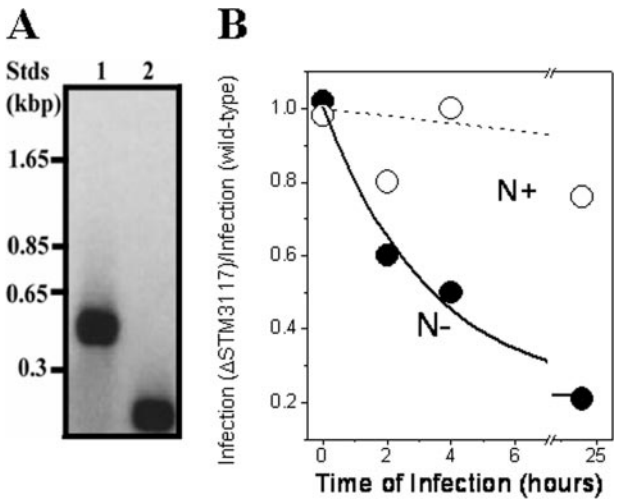

FIGURE 6. **Influence of gene deletion of STM3117 on STM replication in macrophages.** *A*, agarose gel showing PCR products of the STM3117 gene amplified from WT (*lane 1*) and ΔSTM3117 mutant (*lane 2*). The migration positions of standards (*Stds*) are indicated on *left*. *B*, relative viability of ΔSTM3117 cells to WT STM cells upon infection of either N− (●) or N+ (○) macrophages.

DISCUSSION

STM infects macrophages to cause typhoid fever-like diseases in BALB/c mice (1). Although it is broadly recognized that alterations in gene expression patterns are associated with the colonization of macrophages and virulence (23), there have been no comparable measurements of time-dependent changes in protein abundance. To identify key proteins linked to the successful colonization of macrophages, we have isolated STM from RAW 264.7 macrophages derived from BALB/c mice at various times following their infection and used an AMT tag proteomic method to identify 39 proteins whose abundance changes correlate with the establishment of STM colonization in macrophage (Fig. 4). This subset of proteins represents ~12% of the identified STM proteins. Further refinement regarding the identification of critical proteins associated with the infectivity of STM was possible through a comparison of changes in STM protein abundances in response to the presence of Nramp1, a divalent cation transporter in macrophages whose activity results in a substantial reduction of STM virulence. The presence of Nramp1 was found to specifically modulate the protein abundance of six STM proteins (Fig. 5*A*), including known virulence factors such as IHFα, IHFβ, and SodC as well as the proteins Upp, SerA, and STM3117 whose roles in STM pathogenesis had not been determined previously. Deletion of the gene encoding STM3117 resulted in a dramatic reduction in the viability of internalized STM cells within N− macrophages that are sensitive to bacterial colonization (Fig. 6*B*), demonstrating for the first time that the activity of STM3117 represents an important virulence factor that contributes to the replication of STM cells inside macrophages. Based on the putative function of STM3117 and adjacent co-regulated genes encoding proteins associated with peptidoglycan biosynthesis and remodeling, our results emphasize an important role of changes in the structure of the cell wall in the colonization of macrophages by STM.

Our peptide identification of abundant STM proteins are broadly consistent with prior measurements, which have identified abundant proteins for STM bacteria grown in culture under different conditions, as well as for STM isolated from spleen and caecum of infected BALB/c mice (15–20). However, no prior proteomic measurements have been done on either isolated STM from macrophages or measured time-dependent changes in protein abundances associated with the colonization of macrophages. Among the identified STM proteins, two heat shock proteins, *i.e.* DnaK and GroEL (STM4330), were detected previously in the lysates from N− macrophages grown in culture (49). As a virulence factor, DnaK is known to be required for STM survival inside macrophages and contributes to systemic infections in BALB/c mice (49, 50). In addition to DnaK and GroEL, we have identified 39 proteins whose abundance increases after macrophage infection. WB analysis confirmed the increased expression of five of these infection-induced STM proteins. Seven of these induced STM proteins are known virulence factors whose functions range from the regulation of expression of other virulence genes to the sequestration of divalent metal ions essential for STM and detoxification of antibacterial agents produced by host macrophages (34–39). Increased expression of these virulence factors after STM infection of macrophages plays a critical role in promoting the intracellular survival or replication of STM. However, not every STM protein whose expression is induced after infection of macrophages is a virulence factor. For example, PhoN is induced after infection but is not involved in either the replication of STM inside macrophages or in the development of systemic infection in mice (40). Thus, every induced STM protein identified in this study need not be directly involved in the development of STM virulence.

Additional confidence regarding the identification of proteins that are likely to be directly related to STM colonization and virulence involved the identification of proteins whose abundance was dependent on Nramp1 activity. In this respect, prior measurements have demonstrated that the presence of Nramp1 activity in macrophages results in an increased expression of some SPI-2 genes, such as *ompR*, *ssrA*, and *sseJ* (11). Using this strategy, we have identified six new proteins whose abundances are modulated by Nramp1 activity and have no apparent relationship to SPI-2 genes. Three of these Nramp1-affected proteins are known virulence factors (*i.e.* IHFα, IHFβ, and SodCI). Of the three new proteins identified (*i.e.* SerA, Upp, and STM3117), we have focused our attention on the validation and functional importance of STM3117, because this protein has no established function and has not been previously appreciated as a factor in modulating infection of macrophages by STM. WB confirms that the abundance of STM3117, as well as STM3118–3119, increases during STM infection of macrophages and is responsive to the presence of Nramp1 (Fig. 5). Furthermore, deletion of the gene encoding STM3117 reduces the intracellular replication rate of STM in N− macrophages (Fig. 6*B*). These results suggest that STM3117 and STM3118–3119 are new STM virulence factors.

These results are all consistent with prior measurements of gene expression, which demonstrated a coordinated expression

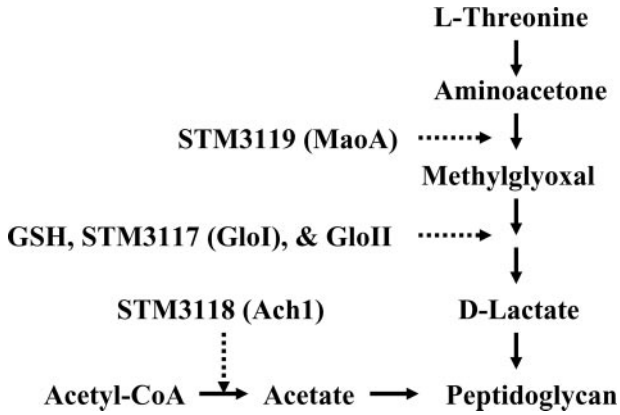

FIGURE 7. **Putative metabolic pathways for STM3117–3119.** *STM3117*, a putative lactoylglutathione lyase (also known as glutathione-dependent glyoxalase I (*GloI*)); *STM3119*, a homolog of monoamine oxidase (*MaoA*); and *STM3118*, a homolog of acetyl-CoA hydrolase I (*AchI*).

of STM3117–3119 that is similar to that for the up-regulation of SPI-2 genes in STM after the infection of N− macrophages (23). Furthermore, peptides of STM3118–3120 have been identified previously from a proteomic analysis of STM isolated from the spleen (but not caecum) of infected BALB/c mice (20), consistent with a functional role for these proteins in mediating macrophage colonization and a systemic infection. These data indicate a fundamental role for STM3117 and neighboring genetic loci in promoting the successful colonization of macrophages necessary for the establishment of a systemic infection.

Although the function remains unknown, sequence comparisons with other known proteins suggest that STM3117 is a putative lactoylglutathione lyase (also known as glutathione-dependent glyoxalase I (GloI)), whose homologs work in concert with GloII to convert highly reactive and toxic methylglyoxal (MG) to D-lactate. Interestingly, STM3119 is proposed to function as a monoamine oxidase (MaoA) that converts aminoacetone, which is the degradation product of L-threonine, to MG (Fig. 7) (51). As a well known fermentation product, D-lactate can also be used as a building block for synthesis of peptidoglycan (52). Furthermore, the putative function of STM3118, a homolog of acetyl-CoA hydrolase I, is to produce acetate, which is commonly used to modify the peptidoglycan layer of the bacterial cell wall to protect bacteria from peptidoglycan degradation enzymes, which are found in macrophages (53). Thus, one possible biological role common for STM3117–3119 is to synthesize and modify the peptidoglycan layer, a major component of STM cell wall that serves as the first line of defense against the antibacterial regents produced by host cells (53). Consistent with this suggestion, prior measurements indicate that extensive peptidoglycan remodeling occurs following proliferation of STM within epithelial cells, and the peptidoglycan layer has been suggested to play an important role in mediating the infection of *Salmonella* (54, 55). Furthermore, the composition of the peptidoglycan cell wall modulates the assembly and function of T3S (56). Thus, our results suggest that abundance changes of STM3117–3119, whose coordinated expression modulates the peptidoglycan layer, function as a critical component in mediating macrophage colonization and subsequent systematic infections.

Acknowledgment—The Pacific Northwest National Laboratory is operated for the Department of Energy by Battelle Memorial Institute under Contract DE-AC05-76RLO1830.

REFERENCES

1. Jones, B. D., and Falkow, S. (1996) *Annu. Rev. Immunol.* **14,** 533–561
2. Richter-Dahlfors, A., Buchan, A. M. J., and Finlay, B. B. (1997) *J. Exp. Med.* **186,** 569–580
3. Salcedo, S. P., Noursadeghi, M., Cohen, J., and Holden, D. W. (2001) *Cell Microbiol.* **3,** 587–597
4. Wijburg, O. L., Simmons, C. P., van Rooijen, N., and Strugnell, R. A. (2000) *Eur. J. Immunol.* **30,** 944–953
5. Fields, P. I., Swanson, R. V., Haidaris, C. G., and Heffron, F. (1986) *Proc. Natl. Acad. Sci.* **83,** 5189–5193
6. Hensel, M. (2000) *Mol. Microbiol.* **36,** 1015–1023
7. Holden, D. W. (2002) *Traffic* **3,** 161–169
8. Knodler, L. A., and Steele-Mortimer, O. (2003) *Traffic* **4,** 587–599
9. Vazquez-Torres, A., and Fang, F. C. (2001) *Microbes Infect.* **3,** 1313–1320
10. Cuellar-Mata, P., Jabado, N., Liu, J., Furuya, W., Finlay, B. B., Gros, P., and Grinstein, S. (2002) *J. Biol. Chem.* **277,** 2258–2265
11. Zaharik, M. L., Vallance, B. A., Puente, J. L., Gros, P., and Finlay, B. B. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 15705–15710
12. Foster, J. W., and Specter, M. P. (1995) *Annu. Rev. Microbiol.* **49,** 145–174
13. Rosenberger, C. M., Gallo, R. L., and Finlay, B. B. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 2422–2427
14. Bader, M. W., Sanowar, S., Daley, M. E., Schneider, A. R., Cho, U., Xu, W., Kievit, R. E., Mousal, H. L., and Miller, S. I. (2005) *Cell* **122,** 461–472
15. Encheva, V., Wait, R., Gharbia, S. E., Begum, S., and Shah, H. N. (2005) *BMC Microbiol.* **5,** 42
16. Qi, S., Moir, A., and O'Connor, C. D. (1996) *J. Bacteriol.* **178,** 5032–5038
17. Yoon, H., Lim, S., Heu, S., Choi, S., and Ryu, S. (2003) *FEMS Microbiol. Lett.* **226,** 391–396
18. Coldham, N. G., and Woodward, M. J. (2004) *J. Proteome Res.* **3,** 595–603
19. Adkins, J. N., Mottaz, H. M., Norbeck, A. D., Gustin, J. K., Rue, J., Clauss, T., Purvine, S. O., Rodland, K., Heffron, F., and Smith, R. D. (2006) *Mol. Cell. Proteomics* **5,** 1450–1461
20. Becker, D., Selbach, M., Rollenhagen, C., Ballmaier, M., Meyer, T. F., Mann, M., and Bumann, D. (2006) *Nature* **440,** 304–307
21. Govoni, G., Canonne-Hergaux, F., Pfeifer, C. G., Marcus, S. L., Mills, S. D., Hackam, D. J., Grinstein, S., Malo, D., Finlay, B. B., and Gros, P. (1999) *Infect. Immun.* **67,** 2225–2232
22. Hashim, S., Mukherjee, K., Raje, M., Basu, S. K., and Mukhopadhyay, A. (2000) *J. Biol. Chem.* **275,** 16281–16288
23. Eriksson, S., Lucchini, S., Thompson, A., Rhen, M., and Hinton, J. C. (2003) *Mol. Microbiol.* **47,** 103–118
24. Elias, D. A., Monroe, M. E., Marshall, M. J., Romine, M. F., Belieav, A. S., Fredrickson, J. K., Anderson, G. A., Smith, R. D., and Lipton, M. S. (2005) *Proteomics* **5,** 3120–3130
25. Smith, R. D., Anderson, G. A., Lipton, M. S., Pasa-Tolic, L., Shen, Y., Conrads, T. P., Veenstra, T. D., and Udseth, H. R. (2002) *Proteomics* **2,** 513–523
26. Paša-Tolic, L., Masselon, C., Barry, R. C., Shen, Y., and Smith, R. D. (2004) *BioTechniques* **37,** 621–639
27. Eng, J. K., McCormack, A. L., and Yates, J. R., III (1994) *J. Am. Soc. Mass. Spectrom.* **5,** 976–989
28. Washburn, M. P., Wolters, D., and Yates, J. R., III (2001) *Nat. Biotechnol.* **19,** 242–247
29. Strittmatter, E. F., Kangas, L. J., Petritis, K., Mottaz, H. M., Anderson, G. A., Shen, Y., Jacobs, J. M., Camp, D. G., II, and Smith, R. D. (2004) *J. Proteome Res.* **3,** 760–769
30. Datsenko, K. A., and Wanner, B. L. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 6640–6645
31. Uzzau, S., Figueroa-Bossi, N., Rubino, S., and Bossi, L. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 15264–15269
32. Rosenberg, C. M., and Finlay, B. B. (2002) *J. Biol. Chem.* **277,** 18753–18762
33. Guignot, J., Caron, E., Beuzon, C., Bucci, C., Kagan, J., Roy, C., and Holden, D. W. (2004) *J. Cell Sci.* **117,** 1033–1045
34. Marshall, D. G., Sheehan, B. J., and Dorman, C. J. (1999) *Mol. Microbiol.* **34,** 134–145
35. Mangan, M. W., Lucchini, S., Danino, V., Croinin, T. O., Hinton, J. C., and Dorman, C. J. (2006) *Mol. Microbiol.* **59,** 1831–1847
36. Blanc-Potard, A. B., Solomon, F., Kayser, J., and Groisman, E. A. (1999) *J. Bacteriol.* **181,** 998–1004
37. Garmendia, J., Beuzon, C. R., Ruiz-Albert, J., and Holden, D. W. (2003) *Microbiology* **149,** 2385–2396
38. Anakiraman, A., and Slauch, J. M. (2000) *Mol. Microbiol.* **35,** 1146–1155
39. Uzzau, S., Bossi, L., and Figueroa-Bossi, N. (2002) *Mol. Microbiol.* **46,** 147–156
40. Miller, S. I., Kukral, A. M., and Mekalanos, J. J. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 5054–5058
41. Navarre, W. W., Halsey, T. A., Walthers, D., Frye, J., McClelland, M., Potter, J. L., Kenney, L. J., Gunn, J. S., Fang, F. C., and Libby, S. J. (2005) *Mol. Microbiol.* **56,** 492–508
42. Kehres, D. G., Janakiraman, A., Slauch, J. M., and Maguire, M. E. (2002) *J. Bacteriol.* **184,** 3159–3166
43. Snavely, M. D., Miller, C. G., and Maguire, M. E. (1991) *J. Biol. Chem.* **266,** 815–823
44. Chakravortty, D., Rohde, M., Jager, L., Deiwick, J., and Hensel, M. (2005) *EMBO J.* **24,** 2043–2052
45. Forbes, J. R., and Gros, P. (2001) *Trends Microbiol.* **9,** 397–403
46. Lam-Yuk-Tseung, S., and Gros, P. (2003) *Cell Microbiol.* **5,** 299–313
47. Peters-Wendisch, P., Stolz, M., Etterich, H., Kennerknecht, N., Sahm, H., and Eggeling, L. (2005) *Appl. Environ. Microbiol.* **71,** 7139–7144
48. Martinussen, J., Glaser, P., Andersen, P. S., and Saxild, H. H. (1995) *J. Bacteriol.* **177,** 271–274
49. Buchmeier, N. A., and Heffron, F. (1990) *Science* **248,** 730–732
50. Takaya, A., Tomoyasu, T., Matsui, H., and Yamamoto, T. (2004) *Infect. Immun.* **72,** 1364–1373
51. Kim, I., Kim, E., Yoo, S., Shin, D., Min, B., Song, J., and Park, C. (2004) *J. Bacteriol.* **186,** 7229–7235
52. van Heijenoort, J. (1998) *Cell. Mol. Life Sci.* **54,** 300–304
53. Boneca, I. G. (2005) *Curr. Opin. Microbiol.* **8,** 46–53
54. Quintela, J. C., de Pedrom, M. A., Zollner, P., Allmaier, G., and Garcia-del Portillo, F. (1997) *Mol. Microbiol.* **23,** 693–704
55. Mouslim, C., Hilbert, F., Huang, H., and Groisman, E. A. (2002) *Mol. Microbiol.* **45,** 1019–1027
56. Pucciarelli, M. G., and Garcia-del Portillo, F. (2003) *Mol. Microbiol.* **48,** 573–585
57. Cherepanov, P. P., and Wackernagel, W. (1995) *Gene* (*Amst.*) **158,** 9–14

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

Liang Shi, Joshua N. Adkins, James R. Coleman, Athena A. Schepmoes, Alice Dohnkova, Heather M. Mottaz, Angela D. Norbeck, Samuel O. Purvine, Nathan P. Manes, Heather S. Smallwood, Haixing Wang, John Forbes, Philippe Gros, Sergio Uzzau, Karin D. Rodland, Fred Heffron, Richard D. Smith and Thomas C. Squier

J. Biol. Chem. 2006, 281:29131-29140.
doi: 10.1074/jbc.M604640200 originally published online August 7, 2006
