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

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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), 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) 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 through 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 phagocytic NADPH oxidase or inducible nitric-oxide synthase, which generate antibacterial reactive oxygen and...
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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

| TABLE 1 | Bacterial strains and plasmids used in this study |
|-----------------|-----------------------------------------------|
| Strains         | Relevant genotype                      | Source or Ref. |
| STM14028        | Wild type                             | ATCC           |
| LS1001a         | + pKD4                                | This study     |
| LS1026          | ΔSTM3117                              | This study     |
| LS1026          | STM45319 + 3 × FLAG                  | 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        |                                          |                |
| pKD4            | A Λ Red (γ, β, and eox)              | 30             |
| pKD4            | kanA                                 | 30             |
| pCP20           | FLP A                                | 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: DYKHDGDDYKHDIDYKDDDK.

dnK 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 trypic digest were purchased from Sigma unless otherwise noted.

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™ Series cell counter (Beckman Coulter, Fullerton, CA), and seeded at 1 × 10⁶ 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 recombiant 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% CO₂. 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 NH₄HCO₃, 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 NH₄HCO₃, 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 twice with the same 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 NH₄HCO₃, pH 8.4. The isolated STM cells were analyzed subsequently by electron microscopy, LC-MS or WB.

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 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...
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 Φ-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′-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.

Table 2
| Primer | Sequence |
|--------|----------|
| 2777TF | ACCGGCGCGCGCTTTATTA7GCQG0TUAGACCGCTCTTATGACTCAAAAGCAGGACGCGG |
| 2777TKR | CCGCCGACTATTGCCTGGCGAAGCGGCGCGCAATGACTCAAAAGCAGGACGCGG |
| 3117TF | GGACCGCTAGAAGACTCTATGCGATTA3GTGACTGAGTCTGCTGACGGCGG |
| 3117TKR | GTGATCGGCAAAGGAGTGGAAATTTCGCAGTATGTTGACTACAAAGACCATGACGG |
| 3119TF | GCAGCAACTTAGAGTAGATGCAAAAAAAATGCATCTGATTGACTACAAAGACCATGACGG |
| 3119TKR | TAACATTTAGTACAGTAAACCAAAACTGCCATTAGTACGAGTACGAACAGCAGGG |
| 3118TF | GTGATCATAATAAGGCAGTAATAGTAAGTTTTAACATTACATATGAATATCCTCCTAG |
| 3118TKR | TAGAGGATCGCTACTTTATGAGACCAGCGGCTTATTATGCCGGGATGACGGCCTGTT |
| 3120TF | GCAGCAACTTAGAGTAGATGCAAAAAAAATGCATCTGATTGACTACAAAGACCATGACGG |
| 3120TKR | TAGAGGATGCTACTTTATGAGACCAGCGGCTTATTATGCCGGGATGACGGCCTGTT |
| 3119TF | TAACATTTAGTACAGTAAACCAAAACTGCCATTAGTACGAGTACGAACAGCAGGG |
| 3125TF | TGAGGATGCTACTTTATGAGACCAGCGGCTTATTATGCCGGGATGACGGCCTGTT |
| 3119TKR | TAGAGGATGCTACTTTATGAGACCAGCGGCTTATTATGCCGGGATGACGGCCTGTT |
| 3118TF | GTGATCATAATAAGGCAGTAATAGTAAGTTTTAACATTACATATGAATATCCTCCTAG |
| 3118TKR | TAGAGGATCGCTACTTTATGAGACCAGCGGCTTATTATGCCGGGATGACGGCCTGTT |

Ethical manipulation 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. 1A). 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. 1B). 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. 2A). 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
proteins relative to those derived from the macrophage host at longer times following infection (Fig. 2C). 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. 1B). 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. 2B). 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. 1B). 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-
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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      |

Consistent decreases in the relative abundance of selected proteins is apparent following the initial colonization of macrophages (Fig. 2D). 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. 4A; 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).

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. 4A) 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.
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. 2A). 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. 5A). IHRα, IHRβ, 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. IHRα, IHRβ, and SodC, have been shown previously to be involved in the development of STM virulence. IHRα and IHRβ 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 (α-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. 5A). A detailed analysis of STM3117 tagged with 3 × FLAG by WB confirms that the abundance of STM3117 is substantially higher in N− cells (Fig.
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**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 STM117 in comparison to Dnak (B) as well as of STM118 and STM119 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.

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

**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. 5A), 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. 6B), 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 sequesteration 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. 6B). 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 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.
Proteomic Analysis of Salmonella Isolated from Macrophages

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