



Top-down proteomics reveals a unique protein S-thiolation switch in *Salmonella* Typhimurium in response to infection-like conditions

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Characterization of the mature protein complement in cells is crucial for a better understanding of cellular processes on a systems-wide scale. Toward this end, we used single-dimension ultra-high-pressure liquid chromatography mass spectrometry to investigate the comprehensive “intact” proteome of the Gram-negative bacterial pathogen *Salmonella* Typhimurium. Top-down proteomics analysis revealed 563 unique proteins including 1,665 proteoforms generated by posttranslational modifications (PTMs), representing the largest microbial top-down dataset reported to date. We confirmed many previously recognized aspects of *Salmonella* biology and bacterial PTMs, and our analysis also revealed several additional biological insights. Of particular interest was differential utilization of the protein S-thiolation forms S-glutathionylation and S-cysteinylation in response to infection-like conditions versus basal conditions. This finding of a S-glutathionylation-to-S-cysteinylation switch in a condition-specific manner was corroborated by bottom-up proteomics data and further by changes in corresponding biosynthetic pathways under infection-like conditions and during actual infection of host cells. This differential utilization highlights underlying metabolic mechanisms that modulate changes in cellular signaling, and represents a report of S-cysteinylation in Gram-negative bacteria. Additionally, the functional relevance of these PTMs was supported by protein structure and gene deletion analyses. The demonstrated utility of our simple proteome-wide intact protein level measurement strategy for gaining biological insight should promote broader adoption and applications of top-down proteomics approaches.

Liquid chromatography-coupled mass spectrometry (LC-MS)-based top-down proteomics is an emerging approach in which whole/intact proteins are separated and fragmented directly in the mass spectrometer to achieve both protein identification and characterization. This is in contrast to the well-established bottom-up LC-MS approaches in which proteins are digested with a protease into smaller peptides and analyzed by tandem mass spectrometry (i.e., MS/MS) to infer protein identity from peptide level information. This approach is presently unsurpassed in its ability to identify large numbers of proteins, but suffers from the loss of information required to distinguish proteoforms (1) that constitute the functionally accurate proteome that is essential for understanding a biological system.

Most top-down measurements have been limited to single proteins or simple protein mixtures due to technical limitations. However, recent advances in instrumentation, separations, and software are now beginning to allow initial applications on much deeper scales. For example, Bungler et al. (2) reported the identification of 322 different proteoforms representing 174 proteins via top-down proteomic analysis of *Escherichia coli* and another study reported identification of 201 different proteoforms representing 154 *Salmonella* Typhimurium proteins by top-down

analysis (3). Top-down proteomics application to bacteria has been infrequent, with a few exceptions such as noted above. Studies with eukaryotic cells have been more prominent and have demonstrated identification and characterization of up to 1,000 unique proteins via top-down means (4–7). For example, Kellie et al. (4) identified and characterized 530 proteins including 1,103 proteoforms in *Saccharomyces cerevisiae*. In a seminal contribution, Tran et al. (7) recently reported the identification of 1,043 proteins from HeLa S3 cells that are dispersed into more than 3,000 proteoforms. More recently Ahlf et al. (8) identified 690 unique proteins and over 2,000 proteoforms from H1299 human carcinoma cells. However, the requirement of customized three- and four-dimensional separation systems in the above studies and large amount of starting material present a potential limitation to the widespread application of high-throughput top-down proteomics.

In the present study, we have used a single-dimension ultra-high-pressure liquid chromatography (UPLC) system coupled with a Velos-Orbitrap mass spectrometer to profile the intact proteome of the Gram-negative bacterial pathogen *Salmonella* Typhimurium (STM). The single-dimension top-down proteomics platform analysis identified 563 proteins including 1,665 proteoforms generated by posttranslational modification (PTMs). Our analysis revealed several intact protein-centric biological insights, while confirming several previously recognized aspects of *Salmonella* biology. Of particular interest, we report the differential utilization of protein S-thiolation forms in *Salmonella* in response to growth under infection-like conditions. Protein S-thiolation is a PTM in which free thiol groups on proteins form mixed disulfides with low-molecular-mass thiols such as glutathione (S-glutathionylation) and cysteine (S-cysteinylation). Protein S-thiolation occurs as a cellular response to oxidative stress, preventing the irreversible oxidation of cysteine residues, as well as under basal (physiological) conditions, in which it can affect protein function, making this PTM crucial for protection and regulation of thiol-containing proteins. Although S-glutathionylation is relatively well studied (see reviews in refs. 9 and 10), less well studied is S-cysteinylation. Literature contains only two

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reports of bacterial S-cysteinylation, both in Gram-positive bacteria (11, 12), and two serial reports on cysteinylation of protein kinase C in mammalian cells (13, 14). Our results report S-cysteinylation in Gram-negative bacteria and report differential utilization of protein S-thiolation forms in a pathogen in response to growth under infection-like conditions. Under infection-like conditions, *Salmonella* preferentially used S-cysteinylation as a mechanism for thiol protection and/or environmental sensing, whereas under basal conditions the pathogen preferentially used S-glutathiolation. These observations were corroborated by complementary bottom-up proteomics data that quantified glutathionylated and cysteinylated peptides. Furthermore, the bottom-up proteomics data in combination with prior transcriptomics data revealed strong induction of cysteine biosynthesis genes under in vitro infection-like conditions, as well as during actual infection of host cells, whereas expression of key glutathione biosynthesis enzymes glutamate-cysteine ligase (GshA) and glutathione synthetase (GshB) were either unchanged or decreased under the same conditions, providing a possible mechanism to explain differential utilization of S-thiolation forms in *Salmonella*.

Results and Discussion

Top-Down Proteomics Analysis and Coverage. A high-throughput top-down proteomics pipeline consisting of a single-dimension Waters UPLC system augmented with long (80-cm) nanocapillary LC columns and an extended (250-min) gradient interfaced with a Velos Orbitrap mass spectrometer was used to profile the intact proteome of *Salmonella* Typhimurium and comprehensively characterize endogenous PTMs and their modulation in response to physiologically relevant conditions. Pathogen growth in Luria-Bertani broth (LB) and in a low-phosphate, low-magnesium, low-pH minimal medium (LPM) represented basal and infection-like conditions, respectively (15–19). In high-resolution (MS and MS/MS), high-throughput mode, the ultrahigh-pressure single-dimension top-down proteomics platform consumed $\sim 5 \mu\text{g}$ of intact protein extract sample per analysis. Top-down mass spectra were analyzed using MS-Align+, an algorithm for top-down protein identification that enables searches for unexpected PTMs (20).

We identified 563 unique *Salmonella* proteins and 1,665 proteoforms at a 5% protein level false-discovery rate (FDR) (4, 7) using the single-dimension top-down platform (Dataset S1). The data represent a broad spectrum of gene products (SI Appendix, Fig. S1) with proteome coverage comparable to the largest top-down eukaryotic datasets reported using three-dimension or four-dimension top-down platforms (SI Appendix, Fig. S2). A number of

factors contributed to the improved top-down proteome coverage using our single-dimension top-down platform compared with prior studies. Among these were the use of an UPLC system with long columns (80 cm) and long gradients (250 min) that afforded efficient protein separation, as well as enough time to limit MS/MS under-sampling, a well-recognized issue for bottom-up proteomics that can be relaxed using long columns and gradients (21, 22). An additional factor is application of the software tool MS-Align+ for top-down protein identification based on spectral alignment, which has been shown to increase the number of identified spectra compared with other currently available tools (20).

Identification of Distinct Proteoforms. The 563 unique *Salmonella* proteins identified in this study are dispersed into more than 1,600 proteoforms created by PTMs. Fig. 1 shows a histogram of the number of identified proteoforms per unique protein, with the majority of the 563 unique proteins being represented by either one proteoform (272 unique proteins) or two proteoforms (101 unique proteins). Twenty-six unique proteins display >10 proteoforms each, with a majority of these abundant primary metabolism and translation machinery proteins. Interestingly, prior work by Manes et al. (23) suggested that certain classes of *Salmonella* proteins, including translation machinery proteins, are preferentially targeted for proteolysis. Proteins in which the start methionine was retained or exhibiting N-terminal methionine cleavage represented $\sim 30\%$ of the proteins identified (Dataset S1). Proteins with evidence of signal peptide cleavage, using the following conservative criteria—presence of the canonical AXA signal peptide motif (24) and considering only the narrow window of proteins with start residue between amino acids 20 and 30—represented $\sim 10\%$ of the proteins identified (Dataset S1).

We found 12 proteins with an N-terminal acetylation modification. In contrast to eukaryotes where the majority of proteins undergo N-terminal acetylation ($>80\%$ in humans), N-terminal acetylation is extremely rare in bacteria. Only five *E. coli* proteins are known to be N-terminally acetylated, including the ribosomal proteins S18, S5, L7, L12, which we identified in our analysis, and elongation factor Tu (25, 26). In addition to the four ribosomal proteins, we also identified eight more bacterial proteins with an N-terminal acetylation modification (Dataset S1) and note that additional N-terminal acetylated bacterial proteins potentially could be identified by using a systematic search. Indeed, Bonnisone et al. (27) analyzing mass spectrometry data from 57 bacterial species suggest N-terminal acetylation is more prevalent than previously appreciated. A total of 20 disulfide-bonded proteins was identified including those previously reported in

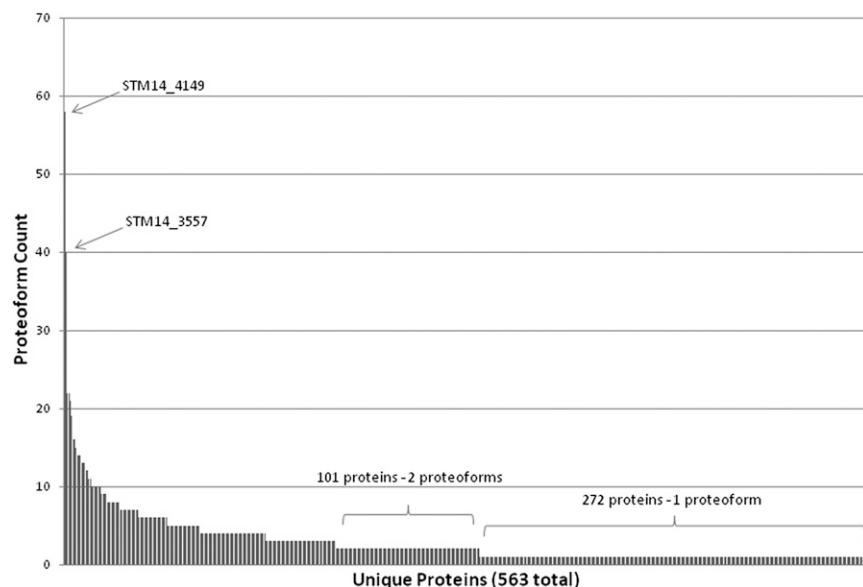


Fig. 1. Analysis of distinct proteoforms. The counts of total proteoforms per unique protein are shown. A majority of entries have only one form identified.

literature such as glutaredoxin (GrxA) (28) and 50S ribosomal protein L31 (RpmE) (29) (Dataset S1). Additionally, three proteins harboring a methylation modification were identified, 50S ribosomal protein L33 (RpmG), 30S ribosomal protein S11 (RpsK), and the elongation factor Tu protein. In *E. coli*, methylation at K56 of elongation factor Tu has been suggested as a mechanism for “fine-tuning” of elongation factor–tRNA intermolecular interactions (30). Our data indicate the same modification at K57 of the *Salmonella* elongation factor protein, the homologous position to K56 of the *E. coli* protein, suggesting a similar functional role in *Salmonella* as in *E. coli*. Other identified chemical modifications included methionine oxidation (14 proteins) and of particular interest protein S-thiolation (25 proteins), which we next discuss in more detail.

Top-Down Proteomics Reveals Differential Utilization of Protein S-Thiolation. From our top-down measurements across basal (LB) and infection-like (LPM) conditions, we identified 25 proteins dispersed into 34 distinct proteoforms having S-thiolation PTMs. Of these, 16 had the +305-Da glutathionylation PTM, whereas 18 harbored the +119-Da cysteinylolation PTM. Interestingly, the glutathionylated proteoforms appeared almost exclusively in the basal LB growth condition, whereas the cysteinylated proteoforms appeared almost exclusively in the infection-like LPM growth condition, which suggests a preferential utilization of glutathionylation in LB and cysteinylolation in LPM. In further support of this notion, we identified a subset of proteins, an example of which is shown in Fig. 2, that exhibited both properties of being

glutathionylated under basal conditions (LB) or cysteinylated under infection-like conditions (LPM) on the same residues, an intriguing switch in modification forms in response to environmental conditions relevant to *Salmonella* Typhimurium pathogen biology. Overall, nine unique proteins exhibited this condition-dependent switch in PTM (see Dataset S2 and SI Appendix, Figs. S3–S10, for details on top-down proteomics data).

Interestingly, structural analysis of this subset of nine proteins revealed that most of the S-thiolation PTM occurred on buried cysteine residues rather than at readily surface accessible cysteines, suggesting this modification is specific and possibly functionally relevant rather than the result of opportune oxidation of the most accessible thiols in the cell (Fig. 3). Calculation of residue accessibility in eight of the nine proteins (or their close homologs in other organisms) for which structures were available using Naccess software (31) showed six of the eight had relative all-atom accessibilities that were less than 20% of that of the reference peptide Ala-Xxx-Ala, and three of these six had less than 5% relative accessibility (SI Appendix, Fig. S11). Further supporting this notion of functional relevance, structural analysis of enolase whose activity is known to be modified by S-thiolation (32) also shows the S-thiolation PTM occurring on a similarly buried rather than surface-exposed residue (Fig. 3). Notably, in two of the proteins, ribosomal protein L3 and glutaredoxin, the S-thiolated Cys is not conserved in the *E. coli* homolog, suggesting that in some cases S-thiolation of these proteins could be a specific adaptation in *Salmonella*.

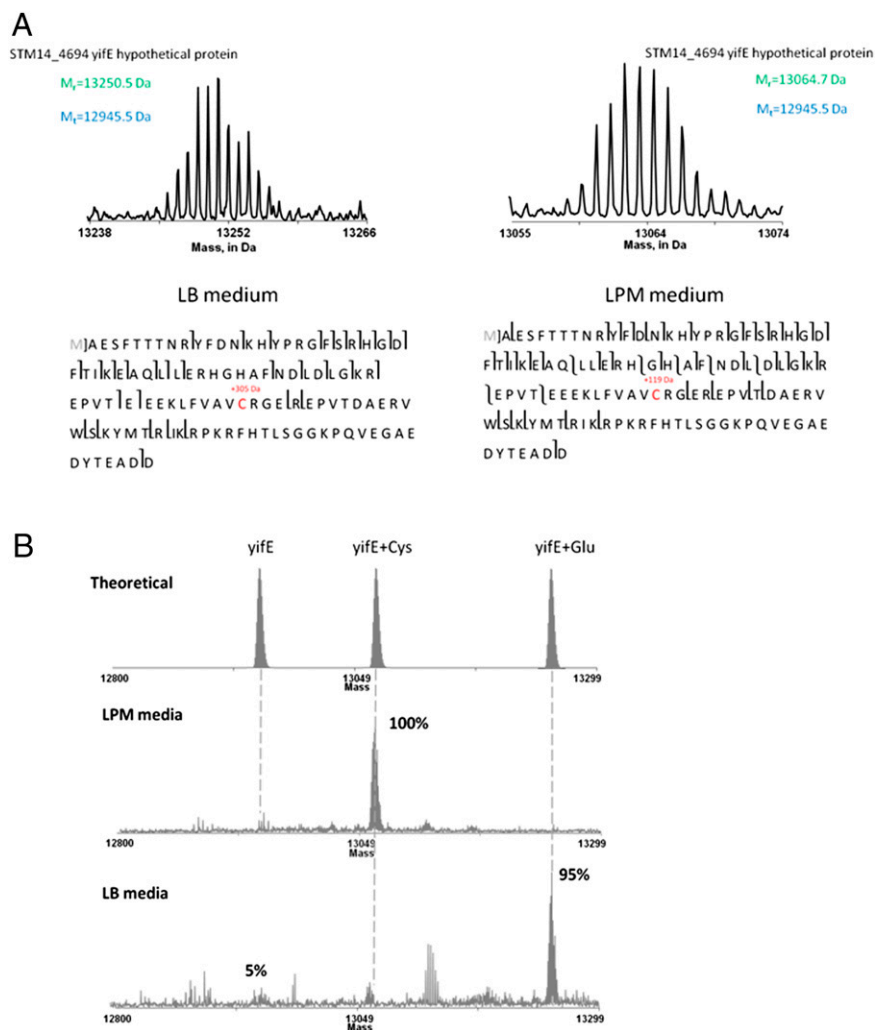


Fig. 2. Switch in modification from glutathionylation to cysteinylolation in response to environmental conditions. (A) Representative zero charge state spectra from top-down proteomics data showing switch in S-thiolation forms in the hypothetical protein YifE (STM14_4694). Fragmentation maps show specific cysteine residue on which switch occurs. (B) Estimated stoichiometry from intact protein mass spectra summed across corresponding LC peak.

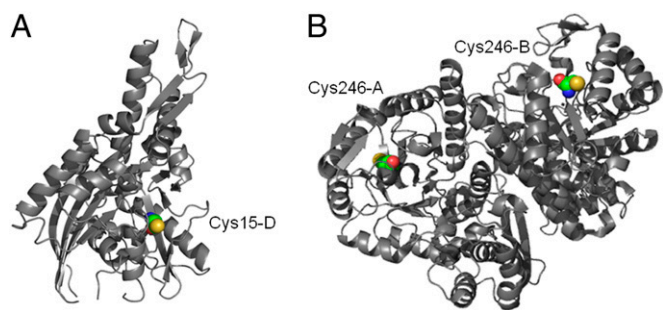


Fig. 3. Structures of *E. coli* homologs of *Salmonella* proteins found to be S-thiolated. (A) DnaK (1kdg). (B) Enolase dimer (1e9i). Modified Cys residues are shown as spheres and the residue number and chain ID indicated. Relative accessibilities of the modified Cys residues calculated with Naccess are 1.3 and 2.1, respectively.

This study reports differential utilization of protein S-thiolation forms, previously unappreciated most likely due to shortcomings of prior methods used (see ref. 33 for review). Briefly, in a typical approach, protein synthesis in the organism under study is inhibited using cycloheximide unavoidably perturbing cell physiology followed by incorporation of radiolabeled cysteine, which does not allow discrimination between the different protein S-thiolation forms, with S-glutathionylation being assumed, and subsequently the system is stimulated with diamide or hydrogen peroxide to stimulate oxidative stress and thus precluding access to proteins glutathionylated or cysteinylated under basal/physiological conditions.

Bottom-Up Proteomics Data Corroborates Top-Down Proteomics Observations. To further validate the observed switch in PTM in

response to physiologically relevant conditions, we reexamined data from an independent bottom-up proteomics analysis of *Salmonella* grown under growth conditions identical to the top-down analysis. The bottom-up proteomics analysis was performed as previously described without any reduction and alkylation (15, 34, 35). Tandem mass spectra were searched against a database of the *Salmonella* Typhimurium proteome allowing for a modification of +305 and +119 on cysteine residues, and the results filtered as described in *SI Appendix, SI Methods*. Examining the global overlap of unique protein identifications between top-down and bottom-up approaches showed 497 of the 563 proteins identified using top-down measurements (88%) were also identified by bottom-up (*SI Appendix, Fig. S12*). Of the 66 proteins not identified by bottom-up, 24 proteins (36%) were <150 aa in length, which may result in fewer ideal peptides for bottom-up analysis.

Next, we used a spectral counting approach to quantify the glutathionylated proteins and cysteinylated proteins in the bottom-up dataset, which revealed 84 unique glutathionylated peptides representing 38 proteins and 62 unique cysteinylated peptides representing 26 proteins. In agreement with the top-down data, the glutathionylated proteins appeared almost exclusively in the basal (LB) growth condition, whereas the cysteinylated proteins appeared almost exclusively in the infection-like (LPM) growth condition, i.e., preferential utilization of glutathionylation in LB and cysteinylation in LPM (*SI Appendix, Fig. S13*). Further analysis of the bottom-up dataset using a peptide intensity-based approach (36) quantified 38 glutathionylated peptides representing 25 proteins and 28 cysteinylated peptides representing 14 proteins (Fig. 4). A comprehensive ANOVA scheme included in the software program DAnTE (37) was used to assess differences in glutathionylated or cysteinylated peptide intensity between LB and LPM in a statistically rigorous manner and fold changes calculated as shown in *Dataset S3*. Consistent with both top-down data and bottom-up spectral count observations, the glutathionylated

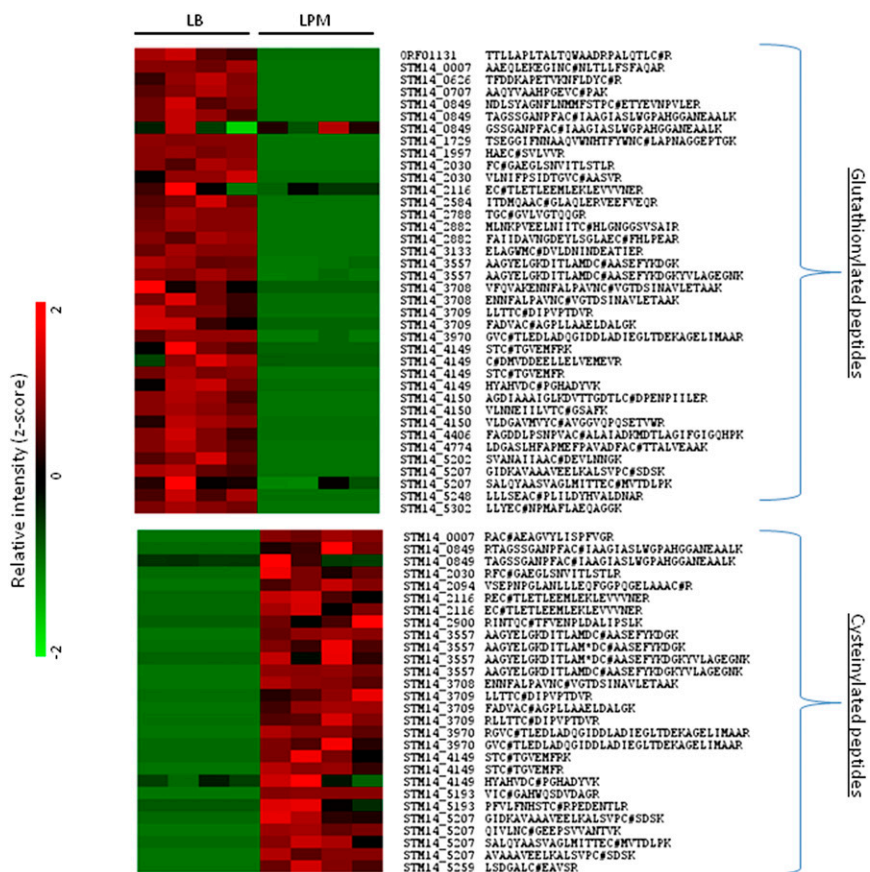


Fig. 4. Quantification of glutathionylated and cysteinylated peptides. Heat map representation of peak intensity-based quantification of glutathionylated (*Upper*) and cysteinylated (*Lower*) peptides identified from bottom-up proteomics data under LB and LPM growth conditions.

peptides/proteins appeared almost exclusively and in high abundance in the basal growth condition [the average fold difference between LB and LPM was ~ 10 -fold ($P < 0.05$)] and the cysteinylated peptides/proteins appeared almost exclusively and in high abundance in the infection-like growth condition [the average fold difference between LB and LPM was ~ 5 -fold ($P < 0.05$)]. Furthermore, a subset of these peptides/proteins exhibited a condition-dependent switch in PTMs as described above (i.e., a glutathionylated protein under basal condition switching PTMs to cysteinylation under infection-like conditions). These included phosphoglycerate kinase (STM14_3709) and elongation factor (STM14_4149), which have previously been reported to be glutathionylated (32) and enolase (STM14_3557) whose activity is known to be modified by S-thiolation (32). To control for the possibility that the abundance increases reported for glutathionylated peptides and cysteinylated peptides above were simply due to just increased protein abundance, we quantified changes in protein abundance using the corresponding non-S-thiolated peptides identified for a representative sampling of the proteins in Fig. 4. As shown in *SI Appendix, Fig. S14*, the switch from LB to LPM does not result in any significant increase in protein abundance, and in some cases there is a decrease in the overall protein abundance, whereas specific S-cysteinylated peptides showed increases in abundance.

We note that validation by Western blot was thwarted by the lack of a commercially available cysteinylated antibody. Furthermore, low sensitivity and specificity of the glutathionylation antibody limits the number of glutathionylated proteins detected under basal conditions (up to four total proteins detected), which constrains their utility for unbiased proteomics studies (33, 38–40). Limited success has been achieved only after stimulating glutathionylation with diamide or hydrogen peroxide and not for detecting endogenous/basal protein S-thiolation as monitored in this study (33, 41, 42). In our hands, the commercially available anti-glutathionylation antibody was not successful in detecting endogenous/basal glutathionylation in *Salmonella*, as has been reported for other systems (see ref. 33 for review).

Proposed Biological Mechanism for the Observed Differential Utilization of S-Thiolation. Literature reports strong induction of cysteine biosynthesis genes for both Gram-negative and -positive bacteria in response to infection-mimicking conditions, including oxidative stress (43, 44). We hypothesized that, under infectious or infectious-like conditions, *Salmonella* uses cysteinylation instead of glutathionylation to protect thiol groups and/or sense the environment in the intracellular compartment, possibly because it is energetically more favorable to synthesize the simpler cysteine moiety or perhaps because it provides faster response to changing environmental conditions.

To evaluate a possible global regulatory hypothesis, we examined the expression patterns of cysteine and glutathione biosynthesis genes under LB and LPM growth conditions (data from this study) as well as in vivo during infection of host cells [data from a previously published dataset (45)]. If the hypothesis were true, then we should observe an up-regulation of cysteine biosynthesis genes in the infection-like condition (LPM) relative to

the basal condition (LB) and, similarly, an up-regulation during infection of host cells, whereas glutathione biosynthesis genes would remain unchanged or may even decrease. Protein expression of glutathione and cysteine biosynthesis genes under in vitro conditions and previously published transcriptomics data of *Salmonella* gene expression during host macrophage cell infection (45) shown in *SI Appendix, Fig. S15*, support this hypothesis. We observed an up-regulation of cysteine biosynthesis genes following infection relative to basal conditions, whereas the glutathione biosynthesis genes showed no significant change in expression in line with our expectation. As a control for a general program of increased biosynthesis required for growth on LPM vs. richer LB media, we also examined the responses for biosynthetic genes for non-S-containing metabolites like nucleotides. Specifically, we examined purine biosynthesis and pyrimidine biosynthesis genes required for nucleotide biosynthesis. As shown in *SI Appendix, Fig. S16 (Inset, Right)*, essentially no response in the 17 nucleotide biosynthetic genes examined was observed for growth on LPM vs. richer LB media. Additionally, we also examined metabolic genes for other S-containing metabolites, specifically methionine and biotin, to further assess specificity of the cellular response. Although methionine and biotin biosynthesis genes appeared to respond similarly to cysteine biosynthesis genes under in vitro conditions (*SI Appendix, Fig. S16, Inset, Left*), under more physiologically relevant in vivo conditions (i.e., an actual infection), only the cysteine biosynthesis genes showed significant up-regulation (*SI Appendix, Fig. S16, main figure*) as would be predicted if the up-regulation being observed were a specific cellular response.

Based on these observations, we have developed an initial model of the differential utilization of protein S-thiolation in *Salmonella* during infection (Fig. 5). Under free living conditions (e.g., in rich media such as LB), there is minimal stress and *Salmonella* can afford to synthesize and use the large tripeptide glutathione for thiol protection and environmental sensing. However, in the harsher intracellular infectious environment, the pathogen must quickly adapt to the change in environmental conditions and reduced availability of nutrients so it synthesizes and uses cysteine rather than the larger tripeptide glutathione. The intriguing possibility that this PTM provides a switch for global regulation of different activities suited to the different environments is particularly intriguing and will be the subject of further study.

Although the essentiality of cysteine biosynthesis genes prevents an assessment of the effect of knockout mutants on *Salmonella* physiology (46), a *Salmonella* Typhimurium mutant strain with a deletion of the important glutathione biosynthesis gene *gshA* was defective in glutathione synthesis, exhibited sensitization to oxidative stress in vitro, as might be expected, as well as a reduction in virulence in mice (47).

Summary

The present analysis using a single-dimension UPLC-MS platform represents proteome coverage comparable to the largest eukaryotic top-down datasets obtained using three or four-dimensional LC-MS platforms. Our global analysis of intact proteins across *Salmonella* basal and infection-like conditions

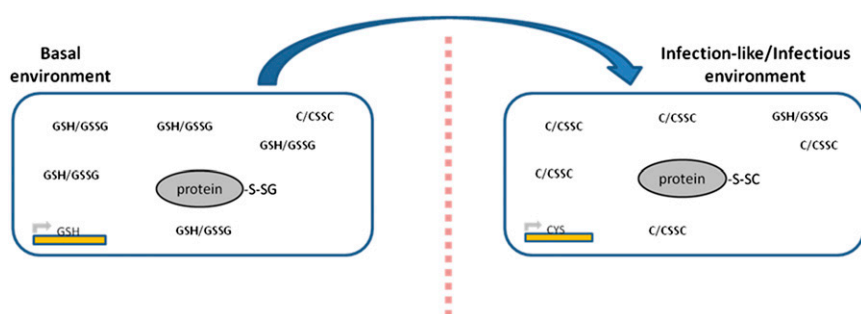


Fig. 5. Working model of differential thiol utilization in *Salmonella* during infection. C, cysteine; CSSC, cysteine disulfide (cystine); GSH, glutathione; GSSG, glutathione disulfide.

confirmed prior observations with respect to *Salmonella* biology and provided additional insights, of particular note the differential utilization of protein S-thiolation forms. The differential utilization of protein S-thiolation forms in response to growth under infection-like conditions corroborated by changes in corresponding biosynthetic pathways reveals a unique protein S-thiolation switch in the bacterial pathogen in response to infection-like conditions and underscores metabolic mechanisms that modulate changes in cellular signaling.

Methods

Sample Preparation. *Salmonella* Typhimurium 14028 was grown in LB medium to log phase or LPM medium for 20 h as previously described (48, 49). Intact protein lysate was prepared by resuspending cell pellets in 100 mM NH_4HCO_3 , pH 8.4, buffer with 1 mM PMSF protease inhibitor and lysed by using 0.1 mM zirconia/silica beads in a 2.0-mL Cryovial with vigorous vortexing for a total of 3 min with cooling steps. Details are described in *SI Appendix, SI Methods*.

LC-MS/MS Analysis. Samples were analyzed using a LTQ Orbitrap Velos MS (Thermo Scientific) coupled to a Waters UPLC platform. Details of the separation and mass spectrometer parameters are described in *SI Appendix, SI Methods*.

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