Global Transcriptome and Mutagenic Analyses of the Acid Tolerance Response of *Salmonella enterica* Serovar Typhimurium

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Salmonella enterica serovar Typhimurium (*S. Typhimurium*) is one of the leading causative agents of food-borne bacterial gastroenteritis. Swift invasion through the intestinal tract and successful establishment in systemic organs are associated with the adaptability of *S. Typhimurium* to different stress environments. Low-pH stress serves as one of the first lines of defense in mammalian hosts, which *S. Typhimurium* must efficiently overcome to establish an infection. Therefore, a better understanding of the molecular mechanisms underlying the adaptability of *S. Typhimurium* to acid stress is highly relevant. In this study, we have performed a transcriptome analysis of *S. Typhimurium* under the acid tolerance response (ATR) and found a large number of genes (~47%) to be differentially expressed (more than 1.5-fold or less than –1.5-fold; $P < 0.01$). Functional annotation revealed differentially expressed genes to be associated with regulation, metabolism, transport and binding, pathogenesis, and motility. Additionally, our knockout analysis of a subset of differentially regulated genes facilitated the identification of proteins that contribute to *S. Typhimurium* ATR and virulence. Mutants lacking genes encoding the K⁺ binding and transport protein KdpA, hypothetical protein YciG, the flagellar hook cap protein FlgD, and the nitrate reductase subunit NarZ were significantly deficient in their ATRs and displayed varied *in vitro* virulence characteristics. This study offers greater insight into the transcriptome changes of *S. Typhimurium* under the ATR and provides a framework for further research on the subject.

Salmonella enterica serovar Typhimurium is a neutralophilic, Gram-negative food- and waterborne pathogen that causes diseases ranging from gastroenteritis to systemic infection in humans. The intestinal tract of wild and domestic animals serves as a vehicle by which salmonellae find their way into humans through contaminated food and water. It has been estimated that globally this species accounts for about 80.3 million cases of food-borne gastroenteritis with about 1.5 million deaths (1). A large number of outbreaks have been linked to contaminated fruits and vegetables, including apples, mangoes, lettuce, tomatoes, celery, and unpasteurized juice (2). During host-pathogen interaction, *Salmonella* constantly encounters various stress conditions, such as changing pH, high osmotic pressure, low oxygen availability, and the presence of bile salts and antimicrobial peptides, that constantly test the adaptability of this pathogen. One such stress condition is low pH, and *Salmonella* confronts this on transit through the stomach, as well as during survival within the *Salmonella*-containing vacuole (SCV) of phagocytic and nonphagocytic cells. Hence, the ability of *Salmonella* to perceive low-pH environments and respond to such stress is crucial for its survival and pathogenicity.

The mechanism by which *S. Typhimurium* senses acidic environments and adapts to survive under low pH is termed the acid tolerance response (ATR) (3–5). The system can be induced by growing *S. Typhimurium* under a mild acidic condition (pH 4.4), called “adaptation,” which provides protection to grow further at a lower pH (pH 3.1), called “challenge,” which is lethal to unadapted cells. Thus, this system usually augments *S. Typhimurium* survival in acidic foods and more importantly under the acidic conditions prevailing in the stomach and SCV. The ATR can be induced during either the log phase or stationary phase of the bacterium, with both systems being functionally distinct (6). There have been a number of studies over the past 2 decades that

focused on different mechanisms involved in the *Salmonella* ATR and its contribution to virulence (4). However, most studies have utilized different conditions for ATR induction, which include various pHs, temperatures, and acids used, and as such, an in-depth model of the *Salmonella* ATR is still lacking. Moreover, because *S. Typhimurium* is a model organism for studying enteric pathogenesis and virulence, it would be valuable to provide a global view of the mechanisms at work during adaptation and survival at low pH.

In this study, we have analyzed the global transcriptome response of *S. Typhimurium* in context of the log-phase ATR in minimal medium using RNA transcriptome sequencing (RNA-seq). This approach revealed global mechanisms employed by *Salmonella* to adapt and survive at lethal low pH and yielded novel candidates whose expression was differentially regulated during the course of the acid response.

MATERIALS AND METHODS

Bacterial strain and growth conditions. Wild-type *S. Typhimurium* strain SB300 was used in this study (7). The strain was grown in minimal

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E glucose (minimal EG) medium containing $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (200 mg), citric acid- H_2O (2 g), K_2HPO_4 (10 g), NaH_2PO_4 (1.75 g), and $(\text{NH}_4)_2\text{HPO}_4$ (1.75 g) in 1 liter of Milli-Q water, adjusted to pH 7.5 with 3 N HCl (8). The medium was supplemented with dextrose (sterilized with a 0.22- μm -pore filter) at a concentration of 0.4%, casein hydrolysate (sterilized with a 0.22- μm -pore filter) at a concentration of 0.1%, and streptomycin (50 $\mu\text{g}/\text{ml}$) to select wild-type *S. Typhimurium*. The log-phase ATR was induced as previously described (6). Briefly, a single colony of *S. Typhimurium* SB300 was grown in 5 ml minimal EG medium or LB, overnight at 37°C and 180 rpm. A 1:100 dilution of the overnight culture was inoculated into 100-ml flasks and incubated until an optical density at 600 nm (OD_{600}) of 0.4 was achieved. Following this, cultures were acid adapted to pH 4.4 (± 0.1) with 3 N HCl and incubated for 60 min. Adapted cultures were subsequently acid challenged by being adjusted to pH 3.1 (± 0.1) with 3 N HCl and incubated for 1, 2, and 4 h postchallenge. CFU were calculated following plating of appropriate dilutions on LB agar. The percentage of viability was determined by dividing CFU at different time points postchallenge by CFU prior to challenge and multiplying the result by 100.

Isolation of bacterial RNA. The ATR protocol was performed in minimal EG medium, and cells were recovered at pH 7.5 (prior to adaptation), 4.4 (1 h postadaptation), and 3.1 (1 h postchallenge). One milliliter of bacterial culture was centrifuged, and the pellet was snap-frozen with liquid nitrogen. RNA was isolated by enzymatic lysis using lysozyme (0.4 mg/ml), set buffer (50 mM NaCl, 5 mM EDTA, 30 mM Tris HCl, 10% SDS), Superase (Thermo Fisher Scientific), mutanolysin (Sigma), and proteinase K (Sigma). The samples were vortexed and incubated at 37°C for 45 min. RNA was isolated using the RNeasy minikit (Qiagen) according to the manufacturer's protocol. During RNA isolation, the samples were treated with RNase-free DNase to avoid DNA contamination. The concentration and integrity of RNA were measured using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and Agilent 2100 Bioanalyzer (Agilent Technologies), respectively. It should be noted that the fragmented 23S rRNA pattern is characteristic of some *Enterobacteriaceae*, including *S. Typhimurium*, and should not be interpreted as RNA degradation. Current algorithms available for assessing quality scores do not take these patterns into consideration and hence indicate false quality scores and incorrect 23S/16S rRNA ratios (9). Only samples having an RNA integrity (RIN) value greater than 6 were used for further workflow. The enrichment of mRNA was achieved by depleting rRNA using the Ribo-Zero Magnetic Gold kit (Epicentre). Two biological replicates were analyzed for a total of 6 (2×3) samples.

Library preparation and sequencing. Fragmentation of the depleted RNA was achieved by incubation with metal ions as per the standard protocol (TruSeq stranded total RNA sample prep kit). Furthermore, fragmented RNAs were primed using random hexamers and Superscript II for first-strand synthesis. Second-strand synthesis was achieved by replacing the RNA template strand, incorporating dUTP in place of dTTP to generate double-stranded cDNA. The cDNA fragments were end repaired, leading to the formation of blunt-end cDNA. The blunt-end cDNA was further adenylated at the 3'-OH end to prevent self-ligation during the subsequent adapter ligation reaction. Adapters with unique indices were ligated to cDNA of the respective samples, following which, a PCR of 15 cycles was performed to enrich the adapter-ligated fragments, thus generating strand-specific libraries with an insert size of 150 to 300 bases. Cleanup between the steps was performed using Agencourt AMPure XP beads. Library quality was assessed on the Agilent high-sensitivity DNA chip prior to being normalized and diluted according to the Illumina MiSeq protocol. Finally, all libraries were sequenced on a flow cell in a multiplexed manner using Illumina v3 chemistry.

Mapping of reads and analysis of differential gene expression. Following image processing/base calling and demultiplexing of reads by Illumina MiSeq-Control software (RTA-versions 1.18.42 and 1.18.54, respectively), read extraction, mapping, and analysis of FastQ-files were performed using the CLC Genomics Workbench version 7.5 (CLCbio).

Sequence reads were mapped against *S. Typhimurium* strain SL1344 (accession no. [FQ312003.1](#)) as a reference sequence (mapping parameters: length fraction and similarity, -80% ; mismatch cost, -2 ; insertion and deletion cost, -3), with the average number of reads mapped to the conditions pH 7.5 (normal), 4.4 (adapted), and 3.1 (challenged) being 6.3, 6.7, and 4.6 million, respectively. Coverage of 85 to 90% of the genome was attained for all runs, with reproducibility of the transcriptome data confirmed by analyzing two biological replicates of each pH condition. The mean fold change value was calculated from the combined replicates of each sample. Genes were considered to be differentially regulated if the average tagwise dispersions $-$ fold change was >1.5 or less than -1.5 , with a false discovery rate (FDR) corrected P value of <0.01 .

qRT-PCR analysis. RNA was isolated as described above, followed by RNase-free DNase I (Fermentas) treatment and cDNA synthesis using the Hi-cDNA synthesis kit (Himedia, India). Quantitative PCR (qPCR) was carried out in duplicate for each sample using the Kapa SYBR Fast qPCR master mix ($2\times$) (Kapa Biosystems, Wilmington, MA) with an appropriate cDNA dilution as the template. The presence of genomic DNA (gDNA) contamination was checked by running a control sample (DNase-treated RNA). The *gmk* (guanylate monophosphate kinase) gene was used as an internal control to normalize the expression of the tested genes. Mean fold change expression values (for each duplicate) were compared.

Generation of deletion mutants and complementing strains. Deletion mutants of target genes were constructed by replacing the gene sequence with a kanamycin (Kan) or chloramphenicol (Cm) resistance cassette using the lambda red recombinase method (10). Briefly, the Kan^r or Cm^r gene was amplified from plasmid pKD3 or pKD4, respectively, using primers that contain regions flanking the target gene to be replaced (see Table S7 in the supplemental material). The amplified products were subsequently transformed into electro-competent wild-type strain SB300 containing the pKD46 helper plasmid required to facilitate homologous recombination. Mutants were selected on LB agar plates containing a suitable antibiotic and confirmed by colony PCR.

The pM2155 vector system was used for the complementation of *flgD* and *yciG* in their respective mutants (11). The genes were amplified with nFPU special DNA polymerase (Enzynomics, South Korea) using primers containing BamHI and HindIII restriction sites at the 5' and 3' ends, respectively. The amplified genes and plasmid were subsequently digested with BamHI and HindIII (NEB), gel purified, and ligated (Fermentas) at 22°C for 1 h. The confirmed constructs (pMflgD for *flgD* complement and pMyciG for *yciG* complement) were complemented into their respective mutants to generate the $\Delta\text{flgD}::\text{pMflgD}$ and $\Delta\text{yciG}::\text{pMyciG}$ strains.

Motility assays. For motility assays, 1.5 μl of bacterial cultures were spotted on soft agar plates (0.3% [wt/vol] agar) prepared in LB medium and incubated at 37°C, for 8 h, following which, the diameter of motile cell growth was measured.

Adhesion and invasion assays. Adhesion and invasion assays were performed as previously described (7). Briefly, HCT116 colon epithelial cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Germany) with 10% fetal bovine serum (FBS) in 24-well plates at 37°C and 5% CO_2 until a confluence of 80% was reached. Prior to infection, culture medium was removed, cells were washed twice with phosphate-buffered saline (PBS), and 500 μl infection medium (without antibiotic) was added. Bacteria were grown overnight in LB medium containing 0.3 M NaCl at 37°C and 120 rpm followed by a 1:20 dilution into fresh medium until an OD_{600} of 0.6. Bacterial counts were obtained by plating serial dilutions on LB agar.

Adhesion assays were performed by keeping both the inoculum and 24-well plates on ice for 15 min prior to infection. Subsequently, cells were infected at a multiplicity of infection (MOI) of 10 followed by incubation for 30 min on ice. For invasion assays, cells were infected at an MOI of 10, followed by incubation for 50 min at 37°C and 5% CO_2 . Cells were then washed twice with PBS, 500 μl infection medium containing gentamicin (100 $\mu\text{g}/\text{ml}$) was added, and the mixture was incubated for 2 h as before.

FIG 1 The acid tolerance response of *Salmonella* Typhimurium. Shown is the log-phase acid tolerance response of *Salmonella* Typhimurium wild-type (WT) strain SB300 in minimal E glucose medium. Both adapted cells (pH 4.4 for 1 h) and unadapted cells were challenged at pH 3.1 (3 N HCl) for 1, 2, and 4 h postchallenge. Bacterial counts were determined at the above time points, and the percentages of viability were calculated for the same time points. Statistical analysis was performed in GraphPad Prism version 6.0, using two-way ANOVA. Statistical significance: ***, $P < 0.001$; ****, $P < 0.0001$.

Postincubation, cells were washed twice in PBS and lysed by adding 500 μ l PBS containing 0.1% sodium desoxycholate, and appropriate dilutions were plated. The percentages of adherence and invasion were calculated by dividing the total number of bacteria recovered by the total number inoculated and multiplying the result by 100.

Macrophage uptake and intracellular survival assay. The intracellular replication assay was performed as previously described (12). Briefly, RAW264.7 mouse macrophage cells were cultured in DMEM supplemented with 10% FBS. Cells were seeded so as to attain a density of 2×10^5 cells per well prior to infection. Bacterial cultures were grown overnight to stationary phase, washed in PBS, diluted to 1×10^8 cells/ml, and infected to obtain an MOI of 10. Following incubation for 50 min in a CO₂ incubator, medium was altered with DMEM containing 100 μ g/ml gentamicin to kill extracellular bacteria. To study uptake, cells were lysed with 0.1% Triton X-100 at 2 h postinfection (p.i.) followed by plating of serial dilutions to determine the number of phagocytosed bacteria. To determine intracellular survival, 2 h posttreatment with 100 μ g/ml gentamicin, macrophage cells were washed twice with PBS, 500 μ l DMEM containing 10 μ g/ml gentamicin was added, and the mixture was incubated for 24 h. The RAW264.7 cells were subsequently lysed and dilutions plated as described above to obtain the number of surviving bacteria. The intracellular survival efficiency was determined as fold increase in the number of bacteria, calculated by the CFU post-24 h versus post-2 h.

Statistical analysis. All experiments were performed in triplicate, unless otherwise stated. Data are represented as the mean $\pm$ standard deviation. One-way and two-way analysis of variance (ANOVA) and *t* test were utilized to determine significant differences. All calculations were performed using GraphPad Prism version 6.0.

RESULTS AND DISCUSSION

Global changes in the transcriptome of *S. Typhimurium* during adaptation and challenge by acidic pH. An analysis of the *S. Typhimurium* ATR, showing survival of both adapted and unadapted cells, revealed a drastic decrease in the viability of the latter up to 4 h postchallenge (Fig. 1). Furthermore, to study the global transcriptome changes of *S. Typhimurium* under the ATR, *S. Typhimurium* SB300 was first grown at pH 7.5 in minimal E glucose (minimal EG) medium and further adapted and challenged at pH 4.4 and 3.1, respectively. This *ex vivo* model of acid tolerance was used as a potential approach to analyze the acid-sensing and acid tolerance mechanism of *S. Typhimurium* (Fig. 2A). Total RNA under the three pH conditions described above

was extracted from flash-frozen cultures, and the rRNA was subsequently depleted. This was followed by sequencing the library preparation as per the TrueSeq sequencing protocol. At each stage, the quality of RNA was determined using the Bioanalyzer 2100. It is interesting to note that the fragmented 23S rRNA pattern that we observed is characteristic of *S. Typhimurium* and should not be mistaken for RNA degradation (see Fig. S1 in the supplemental material) (9). Transcriptional changes in *S. Typhimurium* throughout the course of the ATR were assessed by comparison of the mean levels of expression of sample duplicates between the three pH conditions (Fig. 2B). Within each pH condition, sample duplicates showed a high degree of correlation with Pearson coefficients *r* of 0.995, 0.996, and 0.988 corresponding to pH 7.5, 4.4, and 3.1, respectively (Fig. 2C). In order to impose stringent selection criteria on such vast data sets (see Table S1 in the supplemental material), genes were considered to be differentially regulated if the average fold change value was greater than 1.5 or less than -1.5 with a *P* value of <0.01 . Expression analysis of *S. Typhimurium* during acid adaptation and challenge revealed 2,211/4,742 (46.6%) and 2,325/4,742 (49.02%) genes that are differentially regulated, respectively (Fig. 2D and E; see Tables S2 and S3 in the supplemental material). Functional annotation clustering was performed using David 6.7 (13, 14) and KEGG metabolic pathways. Coregulated genes were identified by the KEGG Pathway database and STRING 9.1 (15). The major groups to which differentially regulated genes were clustered belonged to transport, regulatory functions, pathogenesis, energy, and amino acid metabolism.

ABC transporters and antiporters. Several ABC transporters were differentially expressed during the course of the ATR (see Fig. S2A and Tables S4 and S5). Phosphate transporters such as the *pstSCAB* operon (phosphate uptake) and the *ugpABCE* operon (*sn*-glycerol-3-phosphate uptake) showed upregulation. Both operons have been shown to be induced under phosphate starvation conditions, with the former being regulated by a promoter located upstream of the *pstS* gene and the latter regulated by RpoS, PhoB, and CRP (16, 17). It has also been shown that regulation under phosphate limitation and regulation under acidic conditions are interconnected through RpoS (18). Thus, it is in-

FIG 2 Global changes in gene expression under the ATR. (A) Experimental design. Total RNA was isolated at the indicated pH levels in duplicate. pH 7.5 was used as the reference pH. (B) Hierarchical clustering of differentially regulated genes showing sample duplicates under three pH conditions. Clustering revealed the partitioning of sample duplicates across pH conditions. (C) Sample duplicates displayed a high degree of correlation, with Pearson correlation coefficients ranging from 0.9 to 1. (D) Distribution of mean read counts during adaptation (pH 7.5 versus 4.1). (E) Distribution of mean read counts during challenge (pH 7.5 versus 3.1). Red dots indicate upregulated genes, green dots indicate downregulated genes, and black dots indicate no differential expression.

dicative that under conditions of acid stress (both during adaptation and challenge), the increased metabolic requirement of *S. Typhimurium* would drive the induction of such transporters to allow for ATP production. Another transporter, encoded by *phn-STUV*, importing 2-aminoethylphosphonate (AEP) and part of the operon *phnR* to *phnX*, was overexpressed. AEP can serve as the sole source of carbon, energy, and phosphorus. The operon is induced by the Pho regulon (required for acid tolerance) under phosphate-limiting conditions (19).

The sulfate/thiosulfate transporter encoded by *cysAUW*, involved in extracellular sulfur uptake, showed similar upregulation. In *Escherichia coli*, there are at least four regulators that coordinate sulfur utilization, namely, CysB, MetR, Cbl, and MetJ. Among them, CysB activates associated genes by influencing DNA supercoiling and RNA polymerase binding (20). In addition, it has been shown that CysB is required for the induction of acid tolerance at low pH (21). *S. Typhimurium* CysB mutants have also displayed inhibited binding and uptake abilities along with reduced sulfite reductase activity. In our data set, CysB displayed significant upregulation during acid adaptation and challenge. The high-affinity potassium transporter in *S. Typhimurium* encoded by *kdpABC* showed significant upregulation. pH homeostasis depends on the movement of potassium ions and protons. Under conditions of low extracellular pH, a cell is required to force or pump out ions that are associated with acidification of the cytoplasm. However, due to the potential gradient created by the net inflow of protons, the generated potential gradient prevents further extrusion of protons. This potential gradient is broken up by the active movement of cations such as potassium into the cell, which helps in maintaining the pH balance (22). The *kdpDIE* genes serve as the sensor and transcriptional regulator of the operon and form a two-component system (23).

In addition, two amino acid decarboxylase systems for lysine and arginine encoded by *cadBA* and *adiAC* were significantly overexpressed. These pH homeostatic systems have been shown to consume intracellular protons in response to low pH and export the products, bringing about an increase in intracellular pH (4). Other transporters upregulated exclusively during adaptation included those for proline/glycine betaine transport and cystine transport, while methionine transporters were upregulated exclusively during challenge.

In contrast to upregulated transporters, a number of ABC transporters showed significant downregulation, reflecting the changing requirements of the cell as it transitions from normal to an adapted state. The D-ribose transporter genes *rbsBC* are one such example. The two genes have been shown to be overexpressed in *E. coli slyA* (transcriptional regulator) mutants, which in turn showed an acid-sensitive phenotype, indicating a possible role of *slyA* in acid resistance. Consequently, in our data, *slyA* levels were found to be upregulated during both adaptation (2.8-fold) and challenge (2.5-fold) and hence may be responsible for the downregulation of the ribose transporters. A similar effect has been observed on the *his* operon, which may account for the observed *hisJQ* downregulation (24). The fermentation of sugars such as maltose within the cell generates short-chain acids that, even though excreted, accumulate and reenter the cytoplasm of the cell lowering its pH. Thus, under acid stress, it is expected that sugar transporters, such as those encoded by *malEFGK*, be downregulated (25). Additionally, the *fhuBCD* genes encoding a ferric siderophore transporter were also downregulated. The *fur* gene, in

addition to being a master regulator for iron acquisition, plays a central role in acid tolerance and has been shown to repress the iron-hydroxamate transporter (26). The operon comprising *sfbABC* along with *ompX* constitutes a novel pathogenicity islet of *Salmonella* that is reported to be both iron and pH inducible. However, the genes *sfbABC* showed significant downregulation during both adaptation and challenge, while *ompX* showed no significant change. Moreover, the operon has been shown to be conserved throughout *Salmonella* spp. and may be involved in the import of putative molecules required for virulence (27). Other transporters that were downregulated included those for vitamin B₁₂, spermidine/putrescine, arginine, and oligo- and dipeptides. Taken together, our finding on the differential expression of a large number of ABC transporter families certainly explains their importance in *S. Typhimurium* survival under low pH. Undoubtedly, these ABC transporters are the staff of life for *S. Typhimurium* under acid stress.

Metabolism. During the course of adaptation and challenge with acid, *S. Typhimurium* altered the expression of a large proportion of the genome associated with metabolic pathways (see Fig. S2B and Tables S4 and S5 in the supplemental material). This indicates a rapid coordinated adaptation of the bacterial metabolic processes in response to the acid stress commonly experienced in its natural environment. Several genes belonging to pathways involved in energy metabolism both aerobic and anaerobic showed significant upregulation, including those for glycolysis, the citric acid cycle (tricarboxylic acid [TCA] cycle), and the pentose phosphate pathway (PPP). The *narVWYZ* operon, encoding a second nitrate reductase involved in anaerobic respiration and normally constitutively expressed at low levels (28), showed high expression under both conditions of adaptation and challenge, whereas the major nitrate reductase encoded by the *narGHIJ* operon was significantly downregulated under the same conditions. Genes involved in the electron transport chain (ETC) were also found to be significantly upregulated. Genes encoding several amino acid metabolic pathways were also differentially regulated—in particular, those for arginine, proline, cysteine, methionine, selenoamino acid, thiamine, and tyrosine. Such diverse metabolic upregulation indicates the extensive nutrient requirement of *S. Typhimurium* for adapting and surviving under acid stress.

Regulators. The “bacterial stress response” generally enables bacteria to overcome different stress conditions in their immediate environments. The different mechanisms by which bacteria sense environmental changes are obviously regulated by a complex regulatory system. This complex regulatory system subsequently gives rise to a coordinated and effective response to the respective stress (29). Hence, bacterial regulators are indispensable in mounting an appropriate response against fluctuating environmental conditions, such as pH. In line with the above evidence of the role of bacterial regulators in stress response, we found several *S. Typhimurium* regulators to be differentially expressed under the ATR (see Fig. S2C and Tables S4 and S5 in the supplemental material). The expression profile of RNA polymerase sigma factor RpoS (σ^S) was found to be upregulated under acid challenge. RpoS serves as a regulator during the stationary phase and osmotic and acid shock, as well as regulating the induction of about 10 acid shock proteins that are required for acid tolerance (4). Similarly, the alternative sigma factor RpoE (σ^E) was upregulated under the ATR, and mutants have been shown to

be more sensitive to acid pH as well as having reduced survival in the SCV. Acid activation is shown to be dependent on the processing of the anti-sigma factor RseA by the protease RseP, the latter being essential for acid induction (30).

The *cadC* gene has been shown to play a role as a global translation regulator as well as controlling the expression of *ompR* under acidic conditions. Mutations of *cadC* in *S. Typhimurium* have been shown to enhance survival under acidic conditions by allowing increased expression of *cadA* (31). Interestingly, *cadC* expression levels were found to be downregulated under adaptation but were not significantly expressed under conditions of challenge (−1.3-fold). The ferric uptake regulator, *fur* has been shown to regulate iron acquisition as well as acid tolerance in *S. Typhimurium*, both functions being genetically separable (32). Nevertheless, *fur* expression levels were found to be 1.4- and −1.2-fold during adaptation and challenge, respectively, but were categorized as not differentially expressed (falling within the range +1.5-fold to −1.5-fold). The regulator is required for the induction of a number of acid shock proteins (ASPs): notable among them are genes of the *hyd* (hydrogenase) locus. Broadly, our data fall in line with earlier evidence about the activation of *rpoS* as a transcriptional factor in response to different environmental conditions and *rpoE* an essential transcription factor for exocytoplasmic stress response.

Two-component systems. Two-component systems allow *S. Typhimurium* to sense and respond to stimuli in the environment, each function being performed by a sensor and regulator, respectively. The system serves as the bacterial equivalent of the eukaryotic signal transduction system. The EnvZ/OmpR two-component system has been shown to be involved in the log-phase ATR (6, 33). Expression levels of *ompR* were found to be elevated under both conditions, while *envZ* was overexpressed during adaptation only. Studies have shown that the sensor, EnvZ, responds to low pH by phosphorylating itself and in turn transferring the phosphate group to OmpR. This two-component system plays a central role in the bacterial adaptation to the host, with several downstream effects ranging from proton extrusion and ATP synthesis to regulation of the *Salmonella* pathogenicity island (SPI) genes (34). OmpR has also been shown to regulate another two-component system namely, SsrA/B, which showed high upregulation under both adaptation and challenge. This two-component system is required for the induction of the SPI-2 type 3 secretion system (T3SS), particularly inside the SCV (35, 36). Similarly, the KdpD/E two-component system involved in the regulation of high-affinity potassium transport was upregulated under the ATR. It is responsible for maintaining intracellular osmolarity in response to K^+ limitation in *S. Typhimurium* and has been shown to be induced in an acidic environment in *E. coli* (37, 38). The role of potassium in maintaining pH balance has been further explained in the section on ABC transporters. Other two-component systems that were differentially expressed include the BasS/R system, which has been shown to be induced by acidic or anaerobic growth conditions in *E. coli* (39), and the HydG/H system, which regulates hydrogenase 3 formation and is essential to acid tolerance (40).

As a whole, our analysis of differentially regulated two-component systems during the ATR provides a clear picture about their regulation, as well as the quantitative existence of individual genes in each system. It is interesting to note that although a few two-component systems were investigated earlier in *E. coli* under acid

FIG 3 Expression profile of pathogenicity island-associated genes. Shown is a detailed transcriptional profile of genes that play a role in invasion (SPI1) and intracellular survival (SPI2) of *S. Typhimurium*. Each row represents a gene, with pH levels shown across columns. Fold change values are represented by the color bar alongside the figure. For further details, refer to Tables S4 and S5 in the supplemental material.

stress, their roles in the *S. Typhimurium* ATR remained to be elucidated. Our study has built a framework that can further be utilized to investigate the role of two-component systems in the *Salmonella* ATR at a molecular level.

Salmonella pathogenicity island genes. SPIs are essential for virulence and highly conserved across the genus. Two islands of particular importance are SPI-1 and SPI-2, required for epithelial cell invasion and survival within the SCV of both phagocytic and nonphagocytic cells (41). Both islands encode, in addition to other genes, a type 3 secretion system (T3SS) that is required for the translocation of effector proteins into the cytoplasm of the host that helps to modulate host signaling pathways. Several T3SS genes were found to be differentially regulated under the ATR, including *ssaCG/NQRV* of SPI-2 and *invACE* of SPI-1 (Fig. 3). Additionally, a number of SPI-2 regulators were found to be significantly upregulated, which include SsrA/SsrB, EnvZ/OmpR, and SlyA, indicating a similarity to conditions inside the SCV (42). This result may indeed be correlated with the intracellular survival of *Salmonella*, where acidification of the phagosome serves as a signal for SPI-2 induction. Despite earlier studies revealing the roles of SPI-1 and SPI-2 genes in *S. Typhimurium* survival, our analysis of SPI-1 and SPI-2 gene expression under the ATR serves as a genuine control and validation of our transcriptome experiment.

Flagella and chemotaxis. In the present study, genes belonging to the flagellar assembly (class 2 and 3 operons) (43) and chemotaxis modules were highly downregulated under the ATR (Fig. 4). For all genes observed, there was a greater downregulation during adaptation than during challenge. The alternative sigma factor FliA, resembling sigma 28 of *Bacillus subtilis*, which positively regulates genes for flagellar assembly, motility, and chemotaxis, was also found to be highly repressed (44). It should be

FIG 4 Expression profile of genes involved in flagellar assembly and chemotaxis. The values in the first and second columns represent fold change across the pH conditions indicated at the top of the columns.

noted, however, that the effect of pH on the above genes is poorly understood. An earlier study on *E. coli* revealed low motility to be associated with acid stress (45), while another by Maurer et al. (25) showed flagellar genes to be induced at low pH. Hence, the effect of pH on genes involved in flagellar assembly, chemotaxis, and motility (all of which contribute to virulence) needs to be studied in more detail, particularly with regard to enteric pathogens such as *Salmonella*, which are constantly being exposed to various pH conditions during their course of infection.

Hypothetical proteins and sRNAs. Several hypothetical proteins were highly differentially expressed under conditions of adaptation and challenge. To name a few, the *yciEFG* operon was very highly expressed under the ATR, with *yciG* showing 561.4- and 1,494.6-fold upregulation under adaptation and challenge, respectively. Several others were overexpressed under only one condition: for example, *SL1344_1560* and *SL1344_1200* under adaptation and *yodD*, *yngE*, and *SL1344_0291* during challenge. Interestingly, two pseudogenes, *SL1344_1796* (DNA invertase) and *SL1344_4445A* (hypothetical membrane protein), were also upregulated under adaptation and challenge, respectively, indicating a possible role for them in acid tolerance. A list of hypothetical proteins expressed during the ATR is provided in Table S6 in the supplemental material.

S. Typhimurium expressed 26 small RNAs (sRNAs) during adaptation and 33 sRNAs during challenge, with 21 sRNAs being differentially expressed across the two conditions. The sRNA of *OmrB* was found to be highly upregulated across the ATR conditions and has been shown to regulate the outer membrane protein composition. It is a known negative regulator of *ompT*, *cirA*, and *fecA* and is itself regulated by *OmpR* (46). Another sRNA, *RyhB1*, was differentially expressed under the ATR, with fold values of -3.1 and 2.2 under adaptation and challenge, respectively. *RyhB1* interacts with multiple mRNA targets through *hfq* and has been shown to regulate genes involved in biofilm formation, acid resistance and other stress responses (47). Additionally, the *OxyS* sRNA was overexpressed under both adaptation and challenge, with the latter eliciting a very large fold change. *OxyS* is associated with a

FIG 5 Comparison of gene expression identified by RNA-seq and RT-PCR. The figure compares the mean fold change expression of sample duplicates under the ATR between RT-PCR and RNA-seq. Six upregulated and two downregulated genes were compared under three conditions: normal (pH 7.5), adapted (pH 4.4), and challenged (pH 3.1). *gmk* was used as an internal control, with target expression at pH 7.5 normalized to 1. Both qRT-PCR and RNA-seq data correlated well in terms of determining up- and downregulated genes; however, fold change values showed variation due to significant differences in the sensitivities of the two platforms.

response to oxidative stress and represses *rpoS* (46). In contrast, the sRNA *GcvB* has been shown to be required for acid resistance in *E. coli* through its upregulation of *RpoS* (48). However, interestingly, under ATR conditions, this sRNA was downregulated. Several other sRNAs were differentially regulated under the above conditions, reflecting their roles as important regulators particularly during stress (see Table S6 in the supplemental material).

qRT-PCR validation of RNA-seq gene expression results. To further validate our findings, we analyzed eight differentially regulated genes from the major functional categories (six upregulated and two downregulated) by quantitative reverse transcription-PCR (qRT-PCR). As shown in Fig. 5, qRT-PCR data correlated well with transcriptome sequencing (RNA-seq) data in determining gene up- and downregulation; however, fold change values detected by the two methods showed variation. This is due to the inherent difference in sensitivities of the two techniques in detecting fold change in gene expression.

Identification and characterization of genes that contribute to the ATR of *S. Typhimurium* and their roles in virulence. We hypothesized that highly differentially regulated genes under the ATR might contribute to the survival of *S. Typhimurium* under acid stress. Therefore, we selected 4 genes that showed enhanced expression under the ATR using both RNA-seq and qRT-PCR, encoding proteins belonging to the following functional category:

FIG 6 Characterization of deletion mutants under the ATR and their roles in virulence. (A) Deletion mutants of differentially regulated genes were assayed for their survival under the ATR over a time course of 4 h with wild-type (WT) SB300 as a control. Growth curves of individual mutants and wild-type SB300 over a period of 8 h are shown in the inset. (B) Motility assay of deletion mutants and wild-type SB300. The average diameter (d [$n = 3$]) of motile cell growth in centimeters is shown in the bottom right of each figure. (C and D) Adhesion and invasion assays of the respective mutants and wild-type SB300 in HCT116 colon epithelial cells. The data are presented as the percentage of survival normalized to a wild-type value of 100%. (E and F) Macrophage uptake and survival assay of mutants and wild-type SB300 in RAW264.7 cells. The data represent three independent experiments performed in triplicate. Statistical analysis was performed in GraphPad Prism version 6.0, using t tests and two-way ANOVA. Statistical significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant.

ries: motility (*flgD*), metabolism (*narZ*), transport (*kdpA*), and hypothetical (*yciG*). Knockout mutants of each gene were generated by replacing the coding sequence with a Kan or Cm cassette. The growth rate in LB was determined for all mutants, as several showed growth defects in minimal EG medium. Mutant growth although slightly delayed, was comparable to that of wild-type strain SB300, suggesting that the observed phenotypes were not attributable to growth defects (inset in Fig. 6A). For the same reason and to facilitate a better comparison, the ATR was also performed in LB medium (49, 50) (Fig. 6A). Finally, each mutant was investigated in terms of *in vitro* virulence characteristics, namely motility, adhesion, invasion, uptake by macrophages, and intracellular replication (Fig. 6B to F).

KdpA is a subunit of the KdpFABC potassium-transporting ATPase involved in the binding and transport of K^+ . Intracellular cations, of which K^+ is the most abundant, are absolutely essential for bacterial physiology, and its homeostasis is maintained by three transporters, namely, the high-affinity Kdp transporters and low-affinity Kuk and Trk transporters (51). The growth characteristics, ATR, motility, and macrophage uptake of the $\Delta kdpA$

mutant did not differ significantly from those of wild-type SB300. However, both invasion into epithelial cells and replication within macrophages were found to be significantly reduced, with the latter showing a 3.6-fold replication, as opposed to 42.7-fold replication for SB300. The upregulation of KdpA together with the above phenotypes further supports its role as a gene induced by pH and osmotic stress (52) that plays a role in the virulence of *Salmonella* (51).

NarZ is a subunit of the cryptic nitrate reductase complex NarZYWA involved in anaerobic respiration (53). Previous studies have highlighted the role of NarZ in thermotolerance and acid stress under carbon starvation conditions. Additionally, it has also been shown to be induced within epithelial cells and conditions mimicking the intracellular environment. Consequently, a knock-out mutant was found to be attenuated in a murine virulence model (54). In the present study, in addition to drastically reduced motility, the $\Delta narZ$ mutant mounted a highly weakened ATR, with 0.7% survival at 1 h postexposure to pH 3.1 compared to 8.4% displayed by the wild type. Additionally, adhesion to epithelial cells was significantly lower (1.8%), while no invasion was

FIG 7 Schematic representation of the genes involved in adaptation and survival of *S. Typhimurium* under acid stress. This model represents the transcriptional response of genes belonging to different functional modules (regulation, metabolism, transport and binding, pathogenesis, and motility) of *S. Typhimurium* under the ATR. The genes and pathways mentioned in the Results and Discussion section have been reported. Colors indicate genes that were upregulated (red), downregulated (green), or nondifferentially regulated under both adaptation and challenge (gray) and genes that showed opposing levels of regulation under adaptation and challenge (orange).

observed. Uptake by macrophages did not differ significantly; however, replication was found to be significantly higher than that of wild-type SB300. Thus, it is evident that NarZ is part of a highly regulated, complex network with roles in both ATR and virulence.

FlgD is a scaffolding protein that serves as the hook cap for a growing flagellum (55). The Δ flgD knockout mutant was found to have severely compromised motility and the absence of an ATR phenotype. Adhesion and invasion into epithelial cells were significantly compromised (50.7% and 1.8%, respectively) compared to those of the wild type (100%). Interestingly, uptake by macrophages was about 90-fold higher than that of the wild type, while intracellular replication was 40-fold lower. The increased uptake may possibly be due to an increased contact time between the flgD mutants and macrophages (56), while the deficiency in intracellular replication could be correlated with the highly compromised survival under acid exposure, as indicated above. The

downregulation of this gene under the ATR along with the above phenotypes suggests a role in the survival and adaptation of this bacterium to acid stress and during pathogenesis.

The conserved hypothetical protein YciG may also be involved in the ATR and virulence of *Salmonella*. The Δ yciG mutant displayed significantly reduced motility, which was also reported by Inoue et al. in *E. coli* (57), along with the lack of an ATR phenotype. Adhesion and invasion into epithelial cells were significantly reduced (53.3% and 5.9%, respectively) compared to those of the wild type, while macrophage uptake was about 50-fold greater than that of the wild-type SB300 strain. Interestingly, as with the Δ flgD mutant, the Δ yciG strain displayed a highly attenuated macrophage replication fold in comparison to the wild type. Thus, it would be of particular interest to further elucidate the structure of this small open reading frame and define its functions with respect to acid stress and virulence.

To better characterize mutant strains of interest, the Δ flgD and

$\Delta yciG$ deletion mutants were complemented by inserting a single copy of the corresponding wild-type gene under a constitutive promoter, generating the $\Delta flgD::pMflgD$ and $\Delta yciG::pMyciG$ strains. As shown in Fig. S3 in the supplemental material, all phenotypes were restored in complementing strains, thus confirming the roles of these genes in *S. Typhimurium* acid survival and virulence.

Overall, our knockout analysis revealed the functions of a set of both up- and downregulated genes, showing the utility of a global transcriptomic approach in identifying factors that contribute to stress as well as pathogenesis. The information learned from this subset of genes could further be used to study the mechanisms of molecular interactions that give rise to the observed phenotypes. Mutants such as the $\Delta flgD$ and $\Delta yciG$ strains could further be assessed for their *in vivo* phenotypes and may serve as backbones for live attenuated vaccine development (58).

Concluding remarks. Elucidation of the *S. Typhimurium* transcriptome response during the ATR is vital for a complete understanding of the underlying mechanisms that allow *S. Typhimurium* to survive at low pH. Previous studies on the ATR have been rather fragmented in their approach, and as such, a complete model delineating the global *Salmonella* transcriptomic response during the ATR was lacking. In this study, we characterized the global transcriptional profile of *S. Typhimurium* under the ATR and showed that it undergoes a rapid adaptive response at nonlethal acidic pH, resulting in the remodeling of a large number of pathways involved in transport, metabolism, regulators, motility, and pathogenicity that equips the pathogen to survive lethal pH exposure. A model highlighting our findings is represented in Fig. 7, which provides a summary of the transcriptional response of *S. Typhimurium* under the ATR. We suggest complex regulatory mechanisms are at play in this response, including both coding as well as noncoding elements that coordinate a range of mechanisms and pathways to ensure survival. Our findings on the basis of transcriptional and functional studies show a large group of ABC transporters to be differentially regulated under the ATR. We have also reported that *S. Typhimurium* rapidly adapts to low pH by making significant alterations in metabolic pathways, suggesting a high energy requirement while sensing and mounting a response through various two-component systems. This study has also established a correlation between *S. Typhimurium* ATR and motility, although further work is required to explain the observed gene expression. Most importantly, for the first time, this study has provided a clear physical representation of small noncoding RNA under the *S. Typhimurium* ATR (59) and has identified several functionally uncharacterized proteins.

Mutagenesis and complementation studies were able to identify and confirm the roles of a number of genes, both known and novel, in this *ex vivo* model of acid tolerance as well as to establish a link to virulence. The relationship between acid tolerance and virulence is one of great interest, particularly in the case of enteric pathogens such as *Salmonella*, which must constantly resist pH fluctuation in the gut and the SCV to establish and persist. Thus, acidic pH serves as a signal to trigger an adaptive response that also affects several virulence characteristics. In summary, investigation of the *S. Typhimurium* transcriptome under the ATR provided precious insight into adaptation and survival strategies employed under acid stress. The classical findings obtained by such an approach may help to develop more efficient food storage strategies and therapeutics to combat this resilient pathogen.

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