LWT - Food Science and Technology 92 (2018) 330–338

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Global transcriptomic Acid Tolerance Response in *Salmonella* Enteritidis

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ARTICLE INFO

Keywords:
S. Enteritidis
Acid tolerance response
Transcriptome sequencing
Oxidization damage
Proton balance*Chemical compounds studied in this article:*
Ethidium bromide (PubChem CID 14710)
Hydrochloric acid (PubChem CID 313)
Agarose (PubChem CID 11966311

ABSTRACT

Salmonella is a major foodborne pathogen throughout the world and able to resist extreme acid stress such as stomach gastric juice after mild acid adaptation, which makes it a potential hazard in food industry. We conducted a comparative transcriptomic analysis by high-throughout RNA-seq to elucidate the Acid Tolerance Response (ATR) in *Salmonella* Enteritidis. Among these 554 differentially expressed genes (DEGs) identified in this study, the expression of 280 DEGs were up-regulated and 274 DEGs were down-regulated. First, *S.* Enteritidis tried to survive the adverse environment by reducing the energy-consuming metabolisms and maintaining essential processes as an energy conservation strategy. Second, ATR *S.* Enteritidis underwent damages in DNA, an oxidization damage result in an iron-lacking circumstance and so on. Thus DEGs related to Fe/S cluster biogenesis, stress response regulating proteins and transport proteins were responsive to acid stress to repair damage caused by acid and ROS. Last, to balance extra protons, by increasing the intracellular NAD (+)/NADH ratio, *S.* Enteritidis could also decline the protein acetylation level by promoting the consumption of acetyl coenzyme A via TCA, to prevent the intracellular pH from further decline under acid stress.

1. Introduction

Salmonella is a major foodborne pathogen throughout the world, that causes several diseases in human including fever, abdominal pain, diarrhea, nausea, and sometimes vomiting (Choi et al., 2015). The bacteria are generally transmitted to humans through consumption of contaminated food of animal origin, mainly meat, poultry, eggs and milk (WHO., 2015).

Salmonella has developed very complex regulatory networks responding to various stresses such as extremes of pH (Ren et al., 2015a), heat (Nielsen, Knudsen, Danino-Appleton, Olsen, & Thomsen, 2013), hydrogen peroxide (Kim et al., 2010), hyper-osmosis (Cho et al., 2015) and reactive oxygen (Karavolos et al., 2008). One of the stress responses of concern in *Salmonella* is the Acid Tolerance Response (ATR), which is highly related to food industry where acid is commonly applied to maintain the product quality or prevent microbial contamination. Specifically, *Salmonella* has the ability to survive extreme low pH environment (pH from 2.5 to 4.0) such as stomach acidity if prior adapted to a mild pH (pH from 5.5 to 6.0). Low acid food (pH > 4.6) is very popular and acidic disinfectants are also widely used in contact surfaces cleaning in food industry (O'Leary et al., 2015). Thus mild pH situation could be many acid environments in food processing. Besides mild pH situation also implied as a mixture pH value of neutral food stuff and acid stomach gastric juice (Foster, 1991).

The abilities of pathogens to withstand adverse environmental conditions have long been identified as a presumptive determinant of their virulence potential (Alvarez-Ordonez, Broussolle, Colin, Nguyen-The, & Prieto, 2015). Genes associated with virulence (*hilA*) and invasion (*invA*) shows a higher expression at pH 5 compared to pH 7 with a 24.34 fold and 13.68 fold increase, respectively (O'Leary et al., 2015). Those indicate that stronger virulence might be induced in *Salmonella* when encountering with acid stress.

In the presence of acids, there are a diversity of genes act as global regulations related to a complex tolerance mechanism in *Salmonella*, including *rpoS* and *clpP* (Burin, Silva, & Nero, 2014). *Salmonella* presents similar levels of *rpoS* expression at moderate acidity, that is, pH 5.0 and 6.0 (Burin et al., 2014). Gene *clpP* is also important for the ability of *S.* Typhimurium to grow under low pH and the growth of *clpP* mutant is affected in both an RpoS-dependent and an RpoS-independent manner (Thomsen, Olsen, Foster, & Ingmer, 2002). Some researches illustrated that *ompR* other than *rpoS* was necessary for the global regulation of this adaption response (Ritter et al., 2014; Álvarez-

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https://doi.org/10.1016/j.lwt.2018.02.039
Received 23 December 2017; Received in revised form 12 February 2018; Accepted 15 February 2018
Available online 21 February 2018

Ordóñez, Prieto, Bernardo, Hill, & López, 2012). Lee, Kim, Bang, and Park (2008) also described another regulator linked to the OmpR-EnvZ regulatory system involved CadC in *S.* Typhimurium during acid exposure, which inferred the significance of the lysine decarboxylase system for bacterial survival under lethal acid stress conditions.

The lysine decarboxylase system is composed of a transcriptional regulator of the *cadBA* operon (CadC), a lysine decarboxylase enzyme (CadA), and a lysine-cadaverine antiporter (CadB) (Bang, Audia, Park, & Foster, 2002; Thomsen et al., 2002). Despite of lysine decarboxylase system, there is another prominent amino acid degradation system namely arginine decarboxylase system. It is considered to contribute to intracellular pH stable by pumps (potassium-proton antiporters and sodium-proton antiporters) which extrude protons from cytoplasm at low pH environments (Foster, 2004). Similarly, the arginine decarboxylase system is composed by an arginine decarboxylase (AdiA), an arginine/agmatine antiporter (AdiC) and a transcriptional activator (AdiY) (Kieboom & Abee, 2006).

Moderate acid pH environment would promote the transcription of several PhoP-regulated genes consist of the PhoPQ two-component system and mainly conferring protection to inorganic acid stress (Charles et al., 2009). Ren et al. (2015b) demonstrated that acid stress also led an increase in the intracellular NAD (+)/NADH ratio and down regulation transcriptional level of *pat*, *cyaA* and *crp*. Acid signal also alerted the tricarboxylic acid (TCA) cycle to promote the consumption of acetyl coenzyme A (Ac-CoA), an acetyl group donor for the acetylation reaction. This referred that bacteria could negatively regulate the protein acetylation level to prevent the intracellular pH from further decline under acid stress.

Salmonella could still cause human disease after stomach acidity, which indicates its strong acid resistance and potentials to forming hazard in food. While, the mechanism how *Salmonella* responds to the acid stress environment is still ambiguous. Alexandra Lianou, Nychas, and Koutsoumanis (2017) systematically evaluated the adaptive ATR of multiple *S. enterica* strains, and clearly demonstrated that ATR phenotype was strain dependent. Although several studies have conducted related to ATR in *S.* Typhimurium, the acid response mechanism in *S.* Enteritidis is scarce. We used high throughout RNA-sequencing (RNA-seq) and aimed to describe the major transcriptomic features of ATR in *S.* Enteritidis under stomach acidity compared to neutral pH environment. Followed with a data analysis approach based on direct and functional gene interactions, namely gene set enrichment and cluster analysis, a global transcriptomic ATR in *S.* Enteritidis was elucidated.

2. Materials and methods

2.1. Bacterial strain and stomach acidity imitation

Salmonella enterica subsp. *enterica* serovar Enteritidis ATCC13076 was stocked at −80 °C in Luria-Bertani (LB) with 30% glycerol, and was activated by streaking onto LB plate and incubating at 37 °C for 24 h. *S.* Enteritidis cells from exponential phase (OD600 ≈ 1.0), inoculated in LB liquid media, were collected at 5000 × g for 3 min centrifugation, washed in sterilized phosphate buffer twice, and then resuspended in LB broth up to 6 log CFU/ml. The acid response of acid-adapted and the non-adapted *S.* Enteritidis was evaluated according to the method described in part of a previous study undertaken in our laboratory (Hu et al., 2017). Briefly, for stressed samples, the bacteria in LB broth up to 6 log CFU/ml was subjected to the LB broth (pH of 5.5) for 1 h as acid adaption process and then changed pH value of the broth to final 3.0 with additional HCl (1 M) and maintained for another hour. For unstressed samples, *S.* Enteritidis at the concentration of 6 log CFU/ml was also incubated in LB broth with neutral pH value for 2 h as a negative control group. For the non-adapted sample, *S.* Enteritidis was subjected to an acid LB broth regulated by extra HCl (1 M) to obtain a final pH of 3.0 for 2 h as positive control group. A temperature of 37 °C was maintained throughout the acid adaption or stressed trials and 100 μl samples of each condition were collected every 30 min to determine their cell viability.

2.2. Viability assays and fluorescence microscopy

Viable cell number in the samples was counted with a Live/Dead BacLight bacterial viability kits (Molecular Probes Inc., Eugene, OR, USA) (Zhao, Bi, Hao, & Liao, 2013). The two stock solutions of the stain (SYTO 9 and propidium iodide) were diluted to 3 μl/ml. To determine the standard curve, *S.* Enteritidis cells from exponential phase (OD600 ≈ 1.0), as described above, were harvested by centrifugation at 4000 × g for 3 min, washed in sterilized distilled water twice to a final concentration of 8 log CFU/ml. Bacteria samples were diluted ten-fold with sterile water and blended with 100 μl Live/Dead fluorescence containing SYTO 9 and PI. The mixture was then incubated at room temperature in the dark for 15 min and subjected to Plate Reader according to manufacture's instruction.

For fluorescence microscopy analysis. *S.* Enteritidis cells were collected from 1 ml sample at 5000 × g for 3 min centrifugation, washed in sterilized PBS twice, and then resuspended in 500 μl sterilized PBS. Bacteria samples were blended with equivalent volume of Live/Dead fluorescence containing SYTO 9 and PI. The mixture was then incubated at room temperature in the dark for 15 min (Huang, Quan, Wang, & Chen, 2016) and images were obtained by fluorescence microscopy (Olympus IX73, Japan) with 488 nm excitation (Corich, Soldati, & Giacomini, 2004).

2.3. RNA extraction, library construction

For RNA extraction and isolation, the total RNA of each sample was isolated using the Trizol Kit (Promega, USA) according to the manufacturer's instructions and then residual DNA in the total RNA was removed by treating with RNase-free DNase I (Takara Bio, Japan) for 30 min at 37 °C. RNase free agarose gel electrophoresis and an Agilent 2100 Bio-analyzer (Agilent Technologies, Santa Clara, CA) was used to verify RNA quality. Next, Poly (A) mRNA was isolated using oligo-dT beads (Qiagen). These isolated mRNA was then broken into short fragments in the fragmentation buffer.

For the cDNA library construction, after random hexamer-primed reverse transcription, first-strand cDNA was generated based on the mRNA fragments. RNase H and DNA polymerase I were added to help the synthesis of the second-strand cDNA (Chen et al., 2016). The cDNA fragments were then purified using a QIA quick PCR extraction kit. These purified fragments were subjected to EB buffer for end reparation poly (A) addition and ligated to sequencing adapters. Finally, after agarose gel electrophoresis, these ligated cDNA fragments were extracted from gels and were purified and enriched by PCR to construct the final cDNA library.

2.4. Sequencing and reads alignment

The Illumina sequencing: The cDNA library was sequenced on the Illumina sequencing platform (Illumina HiSeq™ 2000) using the paired-end technology by Gene Denovo Co. (Guangzhou, China). A Perl program were used to remove low quality sequences (that is sequences which has more than 50% bases with quality lower than 20 in one sequence), reads with more than 5% N bases (N means bases unknown) or reads containing adaptor sequences (Chen et al., 2016). SOAPaligner/soap2 is a tool for short sequences alignment which we have applied in a previous research (Hu et al., 2017). These sequencing reads were subsequently mapped to reference sequence by the SOAPaligner/soap2 (Li et al., 2009). All expression data statistic and visualization in this study was conduction with R package (http://www.r-project.org/).

2.5. Differentially expressed genes (DEGs) and function enrichments

For the differential expression genes identification, the bioconductor package edgeR (Robinson, McCarthy, & Smyth, 2010) was applied. The false discovery rate (FDR) was used to determine the threshold of the p value in multiple tests. Specifically, for evaluating whether the gene expression differences were significant or not, a threshold of the FDR ≤ 0.01 and an absolute value of $\log_2$Ratio ≥ 1 were applied (Chen et al., 2016).

The DEGs were then subjected to GO and KEGG enrichment analyses according to previous literature with slight modification (Zhang et al., 2013). Both GO terms and KEGG pathways with a Q-value ≤0.05 in DEGs are significantly enriched in these analyses.

2.6. Quantitative real-time PCR (qRT-PCR) validations

To verify if the gene expression was consistent between RNA-seq and qRT-PCR, 9 DEGs were selected for qPCR analysis using 7500 Fast Real-time PCR System (Applied Biosystem, Foster, USA). The16 S RNA gene of *S.* Enteritidis was used as the internal control gene. Total RNA was extracted with TRIzol reagent according to the manufacturer's protocol, and the RNA was applied to cDNA synthesis with reverse transcriptase. Quantitation of each transcript was repeated using total RNA as the starting materials and each qPCR was performed in triplicate. The primers used for qPCR analysis are listed in Table 1 and some sequences of primers for detection of *S.* Enteritidis were derived from these original ones with modifications.

3. Results

3.1. Effect of acid stress on S. Enteritidis growth

The standard curve was $y = 4.00 \times 10^{-6}x + 553.52$, R-square was 0.9992, y means fluorescence intensity and x means concentration of *S.* Enteritidis cells in CFU/ml. The effect of acid stress on *S.* Enteritidis growth is shown in Fig. 1A. For the negative control group without acid stress, *S.* Enteritidis grew in neutral pH LB broth at 37 °C for 2 h from the original concentration of 6.52±0.05 log CFU/ml to the final of 7.09±0.05 log CFU/ml. For the positive control group without acid adaption, the viability of cells decreased from original concentration to 5.07±0.08 log CFU/ml after 30min, and 4.20±0.06 log CFU/ml at the final. For the ATR group, the viability of cells decreased slightly at the first 30 min and then increase slowly in the following half hour to 6.36±0.03 log CFU/ml when exposed to pH 5.5; the viability of cells decreased to 5.75±0.06 log CFU/ml when exposed to pH 3.0 after 30min. The fluorescence viability was also tested under fluorescence microscope (Fig. 1B), where the green spot indicated viable cells and red spot indicated nonviable cells. In positive control group, at the first 30 min after exposed to pH 3.0, most of cells were destroyed under the extreme acidic shock and damages of cells continued as the time prolonged to 60 min. While, in another condition after pH5.5 acid adaption process, large amounts of cells still alive and their resistance to the following pH 3.0 acid shock were moderately stronger since less death presented at the end of another 60 min acid shock stress. The results were consistent with the plate reader analysis (Fig. 1A). Compared to 96.45% decrease (from 6.52±0.05 log CFU/ml to 5.07±0.08 log CFU/ml) in positive control group at the first 30 min after exposed to pH 3.0, the viability decreased only 75.45% (from 6.36±0.03 log CFU/ml to 5.75±0.06 log CFU/ml) after pH5.5 acid adaption process.

3.2. Data processing and DEGs analysis

Utilizing the Illumina sequencing platform, a great quantity of raw reads was produced and after strict quality control and data filtration, with an average length of 100 bp, 1,476,446,000 and 1,419,719,250 cleaned reads were harvested. After removing contaminated and low-quality sequences, all reads were mapped onto the published transcriptome, which contains 4325 unigenes. In this study, unigenes represented by at least one mapped read were accepted for subsequent analyses. Most unigenes, that is 97.92% and 98.58% of total genes for stressed and unstressed sample, had coverage between 80 and 100%. 4099 unigenes for stressed samples and 4096 unigenes unstressed samples were generated. These basic data of RNA sequencing presented that the data were confidential for further analysis.

DEGs generated from the above unigenes were screened using the threshold of fold-change ≥1 and FDR < 0.05 and 554 DEGs after acid stress (supplementary Table 1) were identified. Among which, 280 DEGs were up-regulated and 274 DEGs were down-regulated, shown in the scatter diagram results (Fig. 2).

3.3. GO analysis and KEGG classification of DEGs

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were used to classify the functions of identified DEGs. For GO analysis results shown in Fig. 3, the height of columns represented the percentage (number) of up-regulated (red) and down-regulated (green)

Table 1
Primer pairs used for qRT-PCR validation.

Gene name	Gene annotation	Primer pair	Reference
16s RNA		5′-AGAGTTTGATCCTGGCTCAG-3′ 5′-ACGGGCGGTGTGTRC-3	(Trkov & Avguštin, 2003)
marA	DNA-binding transcriptional activator MarA	F,5′-CGCAACACTGACGCTATTAC- 3′ R,5′-TTCAGCGGCAGCATATAC- 3′	(Eaves, Ricci, & Piddock, 2004)
soxS	DNA-binding transcriptional regulator SoxS	F,5′-CATATCGACCAACCGCTA- 3′ R,5′-CGGAATACACGCGAGAAG- 3′	(Eaves et al., 2004)
fliC	flagellin	F,5′-GCAGCAGCACCGGATAAAG-3′ R,5′-CATTAACATCCGTCGCGCTAG-3′	(Hong et al., 2008)
rfbS	paratose synthase	F,5′-ACATACTGTGATTGGCTTAG-3′ R,5′-CATTGGCTCTTTCTTTGA -3′	(Ren et al., 2017)
rfbE	CDP-paratose 2-epimerase	F,5′-CTTGGGAGTAATCTTGCC-3′ R,5′-TATACTGCCGTACTGCC-3′	(Itoh et al., 1997)
pyrB	aspartate carbamoyltransferase	F,5′- GAGTACGCCAATGTGAA-3′ F,5′- TGGCGAGCGAAAATACCGTT-3′	(Zhang et al., 2013)
hisG	ATP phosphoribosyltransferase	F,5′- GATGAACGGTGTAGGCTGGAGCTGCTTC -3′ R,5′-CGCCGAAGAACATATGAATATCCTCCTTAG -3′	(Henry, García-del Portillo, & Gorvel, 2005)
ttrC	tetrathionate reductase complex subunit C	F,5′ -ACTGCCGATAAATGCACGTT-3′ R,5′-CTTTTTTCCGCCAGTGAAGA-3′	(Malorny et al., 2004)
dps	DNA starvation/stationary phase protection protein Dps	F,5′-TTATGAGTACCGCTAAAT-3′ R,5′-ATTTATTCGATGTTAGAC-3′	(Kabir et al., 2004)

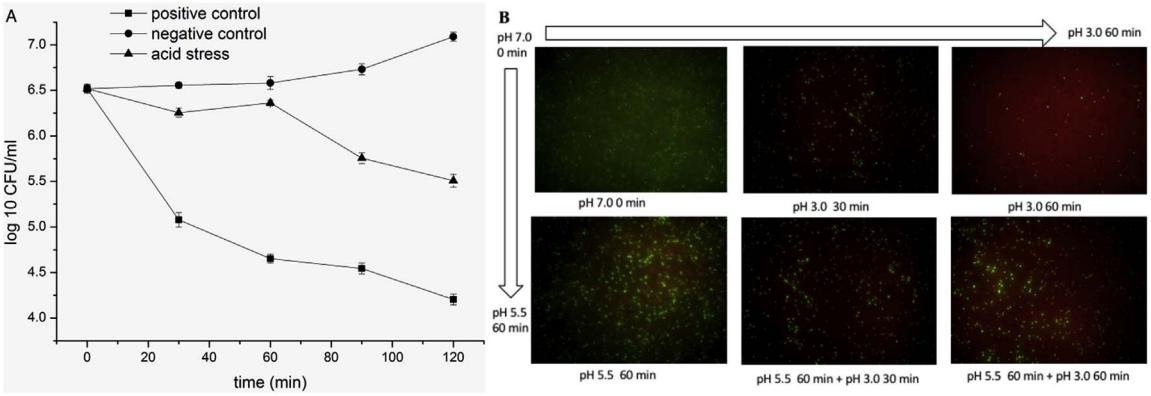

Fig. 1. Growth phenotype of *S.* Enteritidis under acid stress. The green spot indicates viable cells and red spot indicates nonviable cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

genes according to GO database. In the category of biological process, a large proportion of DEGs were related to cellular progress with 77 up-regulated and 101 down-regulated, as well as metabolic progress with 68 up-regulated and 109 down-regulated. Within the category of cellular component, DEGs related to cell and cell part represented the largest clusters since approximately half percentage of DEGs were up-regulated. In the category of molecular function, a high percentage of DEGs was involved in binding with 59 up-regulated and 73 down-regulated and catalytic activity with 70 up-regulated and 118 down-regulated.

KEGG pathway enrichment analysis was also carried out to elucidate the interaction of acid mediated pathways in stress response. The 554 DEGs were successfully matched to 98 different KEGG pathways (data not shown). The pathways significantly changed were listed in Table 2. According to Table 2, pathways associated with flagella assembly, amino sugar and nucleotide sugar metabolism, two-component system, nitrotoluene degradation and histidine metabolism were significantly changed after acid stress.

3.4. Validation of DEGs using qRT-PCR

Several DEGs were selected for qPCR analysis to verify if gene expression was consistent between the qPCR and RNA-seq. The qRT-PCR analysis for 9 genes (6 up-regulated and 3 down-regulated) was used to validate the RNA-seq data in this study. As shown in Fig. 4, qRT-PCR data correlated well with the RNA-seq data (R^2 = 0.8956). Overall, the qRT-PCR data showed similar patterns to those obtained from RNA-seq for these genes, although the particular values of fold-change were diverse with each other.

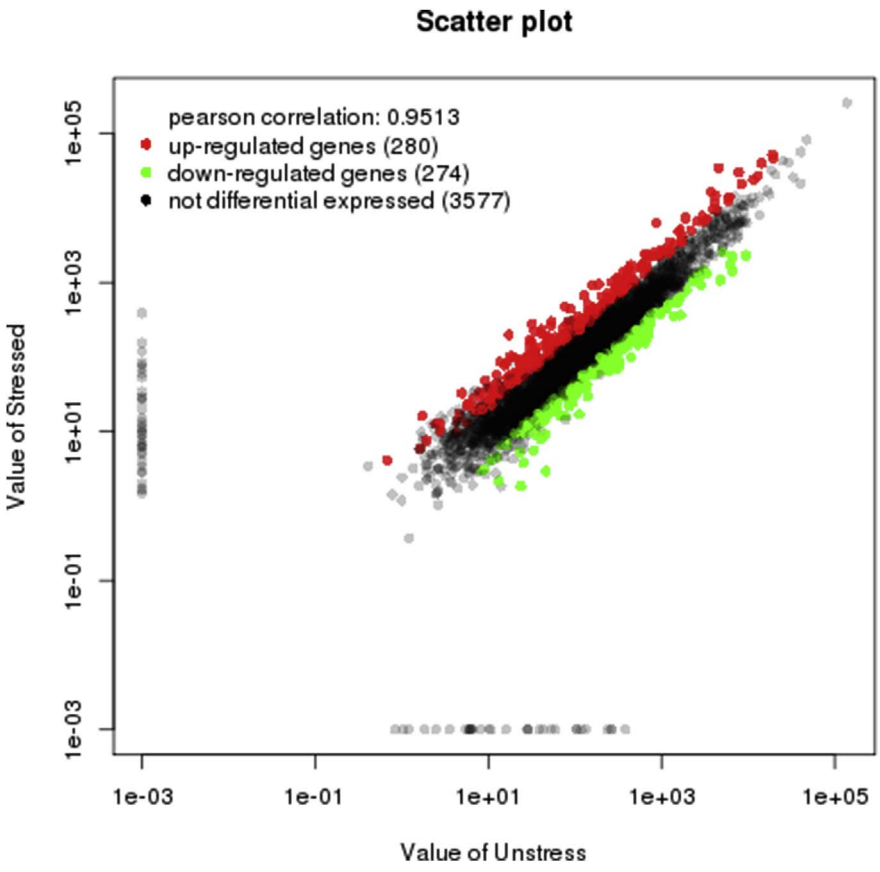

Fig. 2. DEGs between stressed and unstressed *S.* Enteritidis. Red spots represented up-regulated genes, and green spots indicated down-regulated genes. Blue spots represented genes that did not show obvious changes between the stressed and unstressed. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

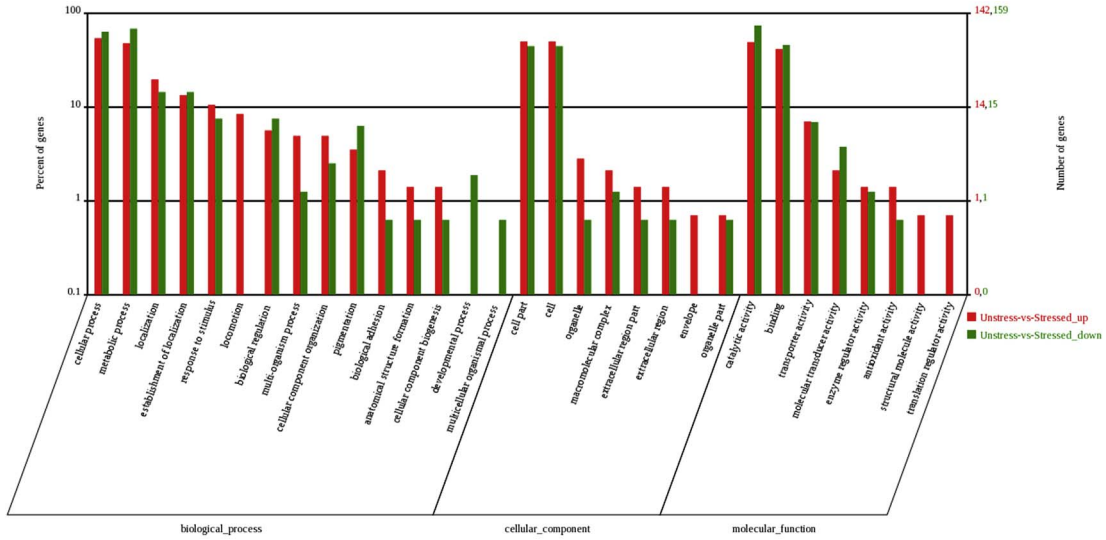

Fig. 3. GO classification of DEG. The *x*-axis indicated the subcategories, the left y-axis represented the percentage of a specific category of DEG and the right *y*-axis indicated the number of DEG.

4. Discussion

Adaptive gene expression allows *S. enterica* to respond to a wide variety of environmental stresses including acidity. In this study, we focused on examining the responses of *S.* Enteritidis to stomach acid stress after acid adaption on transcriptome level. *S.* Enteritidis is a kind of wide spread serotypes of *S. enterica*. We employed a RNA-seq and identified DEGs that potentially mediated the stress response of *S.* Enteritidis. Totally, 554 DEGs after acid treatment was identified, among which 280 were up-regulated and 274 were down-regulated. In this study, combining DEGs identified with functional analysis such as KEGG and GO annotation as well as information in existing literature, we focused on the following aspects associated with ATR from a whole insight.

4.1. Down the energy-consuming metabolism to maintain necessary process

According to KEGG and GO analysis, most genes associated with amino sugar and nucleotide sugar metabolism, which were energy consuming, went through a decreased expression. For example, Gpx and OTCase were down-regulated. Genes such as *caiEF* and *fixC* involved in the pathway of carnitine metabolism and amine and polyamine metabolism were down-regulated. While, the expression of SEN 4285 (type I restriction-modification system specificity subunit M), SEN 4290 (type I restriction-modification system methyltransferase) and SEN 4292 (type I restriction enzyme) increased by 1.34, 2.01 and 1.84 $\log_2$(FC) (FC means fold change), respectively. That indicated that a DNA damage occurred in ATR cells and genes associated with recovery of DNA overexpressed. Cell division protein FtsL ans FtsW down-regulated to prevent cell division and propagation. All these phenomena indicated that cells tried to survive by reducing the energy-consuming metabolisms and maintaining necessary processes.

4.2. Adjustment of secretion systems and other virulence-involved genes

Secretion system Secretion associated DEGs type III secretion protein such as SopE, YscQ, SEN 1645, SEN 1653, and type IV secretion protein VgrEG, SEN 0290 (probable secreted protein, partial) and SEN 1454 (secreted effector protein) were up-regulated. Type III secretion protein is a specialized machine that injects effectors into eukaryotic cells to manipulate the host cell physiology and consist of various genes involved in virulence, adhesion, flagella and secretion (Cascales, 2017).

Virulence-involved DEGs SEN 0277 (exported pathogenicity island protein) was up-regulated 3.23 $\log_2$(FC). *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2) is involved in virulence as well as antioxidant defenses (Fu et al., 2017). Alteration in the virulence characteristics of foodborne pathogens under sub-lethal acid stress conditions was reported previously (Makariti, Printezi, Kapetanakou, Zeaki, & Skandamis, 2015). Gene *gtgA* contributes virulence factors to its host (Ho et al., 2002) was also up-regulated. Besides, gene *rfbS* and *rfbE* were up-regulated under acid stress environment. The *rfb* gene cluster encoded biosynthetic enzymes for the O antigen of *Salmonella* (Brahmbhatt, Wyk, Quigley, & Reeves, 1988; Verma, Quigley, & Reeves, 1988). The abundant expression of *rfb* gene indicated an enhanced virulence under acid stress, since O-antigen was associated with the vitro invasion and virulence (Ilg et al., 2009). The increasing expression of *waaJ* promote the maturation of lipopolysaccharide and O-antigen (Koutsolioutsou, Martins, White, Levy, & Demple, 2001), since O-antigenic polysaccharide of *Salmonella* is both a virulence factor and a protective antigen (Watson, Robbins, & Szu, 1992).

Flagella assembly Under the acid stress in our study, the assembly of flagella basal body operon *flgBCEF* and flagella biosynthesis factor *fliCDFGHI* were up-regulated. FliI and FliK were bound with micromolar affinity. ATP-induced oligomerization of FliI induced kinetic changes, stimulated fast-on, fast-off binding and lowered affinity (McMurry et al., 2015). Wang S. et al. (2010) illustrated that, under

Table 2
KEGG pathway enrichment analyses.

Pathway	DEGs with pathway annotation (374)	All genes with pathway annotation (2849)	Pvalue
Flagellar assembly	19 (5.08%)	59 (2.07%)	0.0001032378
Amino sugar and nucleotide sugar metabolism	18 (4.81%)	78 (2.74%)	0.01023806
Two-component system	36 (9.63%)	196 (6.88%)	0.01915658
Nitrotoluene degradation	4 (1.07%)	11 (0.39%)	0.04531115
Histidine metabolism	6 (1.6%)	21 (0.74%)	0.04753074

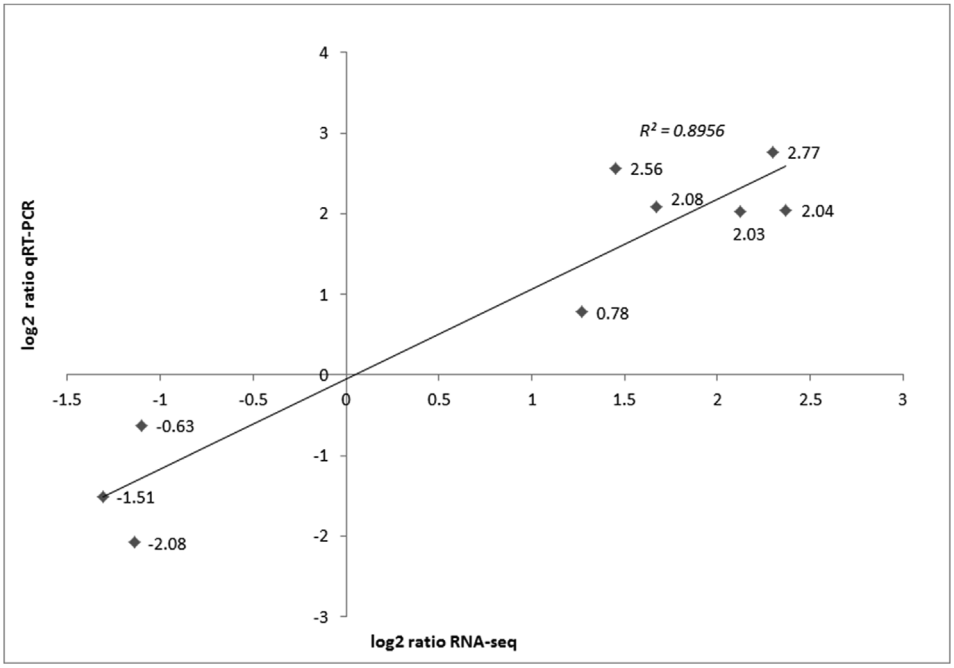

Fig. 4. qRT-PCR validation. The horizontal x-axis indicated the log2 fold change according to RNA-seq, the vertical y-axis represented the log2 fold change according to qRT-PCR, the R^2 means the R-square of regression line.

chlorine oxidation, the flagella basal body operon *flgBCDEFG* and flagella biosynthesis factors such as *fliA* and *fijB* were down-regulated. It is said that inhibition of the synthesis and assembly of flagella, which are large macromolecular complexes of the bacterial cell, may serve as an energy conservation strategy for *S. enterica* under chlorine oxidation stress. While this is contradictory to cells' response in acid stress according to our results since the flagella biosynthesis related genes were unregulated. A functional flagellum was required for epithelial cell invasion and macrophage uptake, probably in a motility-independent mechanism. While, flagella were found to be dispensable for host cell adhesion (Elhadad, Desai, Rahav, McClelland, & Gal-Mor, 2015). Although the synthesis of flagella increased, its motility has not increased which determined by gene *motA* and *motB*. The increase in the synthesis of flagella in its adaptation to stress probably accelerated its colonization and invasion ability.

4.3. Restriction of proton transport from extracellular with two-component systems

Histidine metabolism involved genes *hisG* (EC:2.4.2.17), *hisA* (EC:5.3.1.16), *hisB* (EC:4.2.1.19, EC:3.1.3.15), *hisC* (EC:2.6.1.9) and *hisH* were down-regulated, which control the consuming of PRPP from Pentose phosphate pathway to L-Histidine. Two-component signal transduction systems enable bacteria to sense, respond, and adapt to a wide range of growth conditions and usually consist of a sensor histidine kinase and its cognate response regulator. Several two-component systems in *S. enterica* were involved in acid resistance. ***DEGs associated with CitB family***. CitA sensor histidine kinase decreased (−1.16 $\log_2$(FC)) to prevent extra citrate transport inside and CitC (1.68 $\log_2$(FC)) and CitX (1.65 $\log_2$(FC)) increased to promote the consuming of acetate and citrate inside cells through citrate fermentation. ***DEGs associated with OmpR family***. Among the OmpR family, gene PstS, OmpC, FlhC and FliA were up-regulated; gene RtsB, BaeS, BasR and AmB were down-regulated. Ellermeier & Slauch, 2003 showed that RtsA and RtsB coordinate induction of invasion and repression of motility in the small intestine. RtsB represses expression of the flagellar genes by binding to the *flhDC* promoter region and subsequently decreased expression of the entire flagellar regulon. Gene *rpoS* was necessary for stress adaption response, which was identical to previous researches (Ritter et al., 2014; Álvarez-Ordóñez et al., 2012). In our study, FliZ, a RpoS antagonist and putative regulator of FliA activity, abundance during the ATR process. FliA positively regulated *flhDC* and subsequently activated FliZ (Sim et al., 2017). ***DEGs associated with Chemotaxis family***. As for the Chemotaxis family, CheY and MCP were up-regulated. MCP is associated with attachment. The increase of CheY which is associated with Flageller motor switch adaption would enhance invasiveness ability (Jones, Lee, & Falkow, 1992).

4.4. Acidic environment stabilizes ferrous Fe

In our study, most ABC transports subfamilies that controlled the transportation of molecular were down-regulated despite of some DEGs as involved in sulfate metabolism or iron complex maturation. Up-regulation of iron acquisition systems to promote bacterial survival under H_2O_2 pressure has been reported previously (Fu et al., 2017) and this is consistent with our results. Fe/S proteins function in diverse biological processes (Roche et al., 2013) and [Fe-S] cluster contains acid-labile prosthetic groups (Johnson, Dean, Smith, & Johnson, 2005). Specifically, high intracellular levels of unbound iron might contribute to the production of reactive oxygen species (ROS) via Fenton reaction and increased ROS levels lead to damage of proteins with [Fe-S] cluster vice versa (Strzyz, 2016). Thus, organisms have to coordinate and balance their responses to oxidative stress and iron availability. Environmental stress including acidity could stimulate the accumulation of ROS in cells that has the ability to cause antioxidant damage (Xiang, Hu, Hu, Pan, & Ren, 2015).

According to our results, SufD, Suf, SufB, SEN 1676 (iron-sulfur cluster assembly scaffold protein) were up-regulated dramatically responding to acidity. The iron-sulfur cluster (ISC) and sulfur mobilization (SUF) systems carry out biogenesis and maturation of all Fe/S clusters in *Salmonella* (Takahashi & Tokumoto, 2002). In the SUF system, SufSE forms the Fe/S cluster, and SufBCD complex is responsible for cluster transfer and release. SUF system was induced under anaerobic or iron-limited environment (Takahashi & Tokumoto, 2002). Gene *cysW*, SEN 2261 (2Fe-2S ferredoxin), Fep, gene *fhuD*, SEN 3272 (bacterioferritin-associated ferredoxin), gene *sitABCD* were

unregulated. These phenomena confirmed that ATR *S*. Enteritidis went through an oxidization damage or iron-lacking circumstance.

SEN 0287 (LysR family transcriptional regulator) was up-regulated, and LysR family transcriptional regulator was considered to be involved in oxidant-resistance (Åslund, Zheng, Beckwith, & Storz, 1999). Activated OxyR could induce transcription of a set of antioxidant genes, including *katG*, *ahpC*, *dps* and *oxyS* (Remes, Berghoff, Förstner, & Klug, 2014). According to our results, the expression of SoxS was up-regulated 1.27 $\log_2$(FC). Superoxide-generating compounds, activate the transcription factor SoxR by oxidizing the 2Fe-2S cluster and oxidized SoxR then induces the expression of the second transcription factor SoxS (Zheng, Doan, Schneider, & Storz, 1999). The up-regulation of SoxS confirmed the hypotheses that ATR *S*. Enteritidis went through oxidization damage.

Down regulation of TrrBC indicated that the maturation of Fe-S cluster was defect in some extent. TtrA and TtrB are predicted to be anchored by TtrC to the periplasmic face of the cytoplasmic membrane implying a periplasmic site for tetrathionate reduction. Specifically, TtrA contains a cofactor and a [4Fe-4S] cluster, that TtrB binds four [4Fe-4S] clusters, and TtrC is an integral membrane protein containing a quinol oxidation site. (Hensel, Hinsley, Nikolaus, Sawers, & Berks, 1999).

As a result of the same defect, the activity of enzymes need Fe/S cluster were affected. Gene *hybG*, *hybE*, *hybD*, *hydB*, *hyaC*, *hybA*, *hydA* and SEN2241 (quinol dehydrogenase periplasmic component) which in need of the maturation of Fe/S cluster as active site were down-regulated indicated a depression of aerobic respiration (Soboh et al., 2013). Gene *cydB* and *cydA*, which catalyzed the reduction of oxygen to water and in need of Fe/S cluster, were also down-regulated.

4.5. Alteration in TCA cycle

SEN 1498 gene *patB* was down-regulated in the ATR environment, which is consistent with previous report. Gene *metAFK* and SEN 3022 (NADPH-specific quinone oxidoreductase) were up-regulated. Ren et al. (2015b) demonstrated that acid stress also led an increase in the intracellular NAD (+)/NADH ratio and down-regulated the transcriptional level of *pat*. As Fig. 5 showed, intracellular NAD (+)/NADH ratio increased for the upregulation of *mdaB* and SEN 4159. Bacteria could also decline the protein acetylation level by promoting the consumption of acetyl coenzyme A (Acetyl-CoA), to prevent the intracellular pH from further falling under acid stress. Gene *prpBC* associated with TCA and gene *ribH* associated with production of FMN and FAD was up-regulated. Gene *ulaDE* and *ulaG* associated with L-ribulose-5P were differentially expressed.

4.6. Regulation of other stress responding proteins

Some stress-induced proteins such as phage shock protein (*pspAB*), SEN1385 phage membrane protein, SEN1399 multi-drug resistance, heat shock protein (*ibpAB*) MdtJ (multidrug efflux system protein) and SEN 1800 (heat shock protein) exploded during the ATR process.

Rof (Rho-binding antiterminator) increased the efficiency of transcription by appropriate recognition of the promoter site. Regulation of gene expression by premature termination of transcription, or transcription attenuation, is a common regulatory strategy in bacteria (Henkin & Yanofsky, 2002). In our results, transcription antitermination protein NusB and SEN1139 (phage antitermination protein Q), Multiple stress resistance protein BhsA and DNA starvation/stationary phase protection protein Dps were also up-regulated.

HNH endonuclease domain-containing protein promote the homing of genetic elements into allelic intronless or inteinless sites (Carr, Penfold, Bamford, James, & Hemmings, 2000) was up-regulated 2.73 $\log_2$(FC) indicated that duplication and horizontal transfer events enhanced.

Gene *marAB* were significantly upregulated in our study. The expression of *marA* of the multiple antibiotic resistance (*mar*) locus or of the *soxS* or *robA* gene product produced tolerance to cyclohexane (White, Goldman, Demple, & Levy, 1997).

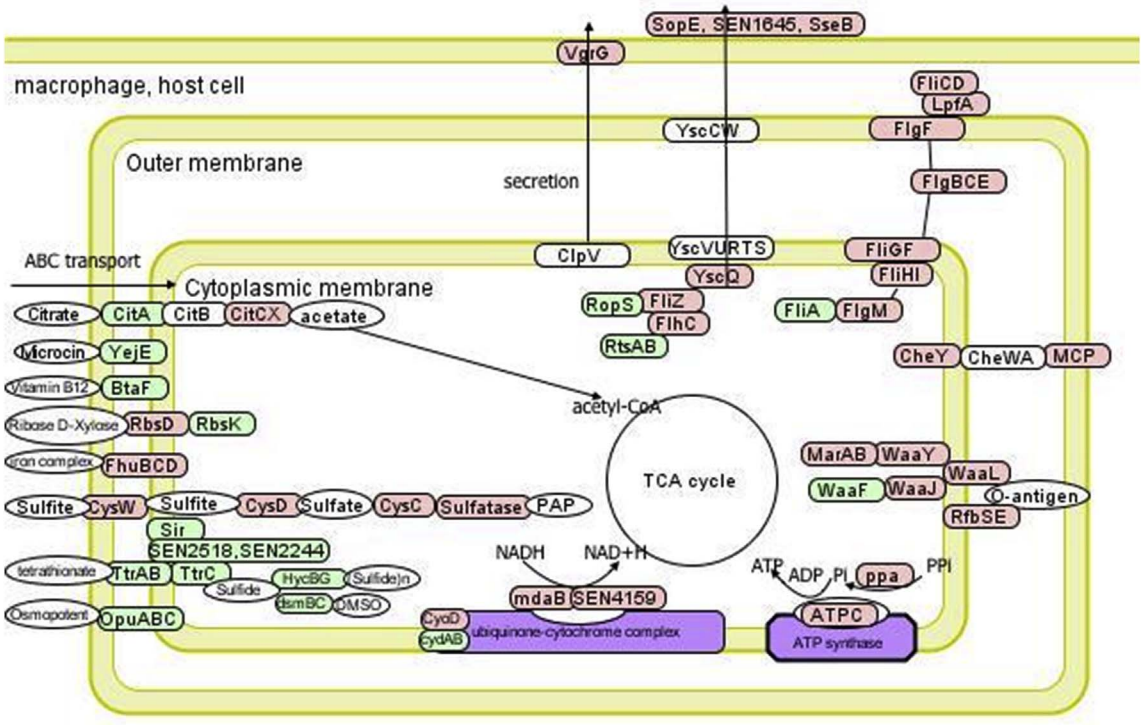

Fig. 5. Global gene regulation in ATR *S*. Enteritidis. Circle means molecular or intermediate and red and blue bar mean gene up-regulated and down-regulated, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

5. Conclusion

To summary, in this study, comprehensive genomic information was obtained about the ATR in *S.* Enteritidis by using throughput transcriptome sequencing technology. Among these 554 DEGs, identified between the stressed and unstressed samples, 280 were up-regulated and 274 were down-regulated. In spite of excessive protons, ATR *S.* Enteritidis went through an oxidization damage leading to iron-lacking circumstance, DNA damage and so on. **1)** various functional gene categories including those related to Fe/S cluster biogenesis, stress response regulating proteins and transport proteins were responsive to ATR system in *S.* Enteritidis to prevent damage caused by acid and ROS. As a result of the iron-lacking defect, the activity of enzymes needed Fe/S cluster were affected and iron acquisition systems were up-regulated at the same time to promote bacterial survives. **2)** The colonization and invasion abilities and virulence were accelerated. Cells tried to survive by reducing the energy-consuming metabolisms such as the synthesis of some compounds and maintaining necessary processes such as repair of DNA damage. **3)** To balance extra protons, by increasing the intracellular NAD (+)/NADH ratio, *S.* Enteritidis could also decline the protein acetylation level by promoting the consumption of acetyl coenzyme A via TCA, to prevent the intracellular pH from further falling under acid stress.

Acknowledgements

Funding

This study was funded by Science and Technology Program Foundation of Guangdong Province (grant number 2013A090100014, grant number 2015A030401025 and grant number 2016B090918103), Natural Science Fund of Guangdong (grant number 2016A030313449), National Key Research and Development Program of China (2016YFF0203204) and Fundamental Research Funds for the Central Universities (grant number 2015ZZ123). We thank Guangzhou Gene Denovo Biotechnology Co., Ltd., China, for technical assistance.

Conflict of interest

Shuangfang Hu declares that she has no conflict of interest. Yigang Yu declares that he has no conflict of interest. Donggen Zhou declares that he has no conflict of interest. Rong Li declares that he has no conflict of interest. Xinglong Xiao declares that he has no conflict of interest. Hui Wu declares that he has no conflict of interest.

Ethical approval

This article does not contain any studies with animals performed by any of the authors.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.lwt.2018.02.039.

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