Global transcriptomic Acid Tolerance Response in Salmonella Enteritidis

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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-throughput 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 oxidation 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 (Kraravolos 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-Ordóñez, Broussolle, Colin, Nguyen-Thé, & 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-Ordóñez, Broussolle, Colin, Nguyen-Thé, & Prieto, 2015).

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6 log CFU/ml was subjected to the LB broth (pH of 5.5) for 1 h as acid
negative control group. For the non-adapted sample, stressed samples,
with additional HCl (1 M) and maintained for another hour. For un-
was maintained throughout the acid adaption or stressed trials and
subjected to an acid LB broth regulated by extra HCl (1 M) to obtain a

2.1. Bacterial strain and stomach acidity imitation

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
Salmonella enterica strains, and clearly demonstrated that ATR pheno-
type 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 throughput RNA-sequencing (RNA-
seq) and aimed to describe the major transcriptomic features of ATR in S.
Enteritidis under stomach acidity compared to neutral pH environ-
ment. 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. Salmonella 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 distilled water twice to a nal pH of 3.0 for 2 h as positive control group. A temperature of 37 °C

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 manufacturer's instruction.

For fluorescence microscopy analysis. S. Enteritidis cells were col-
lected 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 incu-
bated at room temperature in the dark for 15 min (Huang, Quan, Wang, & Chen, 2016) and images were obtained by fluorescence micro-
scopy (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 manu-
facturer's instructions and then residual DNA in the total RNA was re-
moved by treating with RNase-free DNase I (Takara Bio, Japan) for
30 min at 37 °C. RNase free agaroase 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 frag-
ments 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 agaroase 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 pro-
gram 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). SOAPa-
ligner/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). The 16S 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 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)
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.
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 adaptation 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 view.

### 4. Discussion

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 *catEF* and *fxC* 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 log₂(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 *PtsL* and *PtsW* 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.

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

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### 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 log₂(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 *gtxA* 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 *waaL* 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 *flaBCEF* and flagella biosynthesis factor *flaCDFGH* were up-regulated. *FlaI* and *FlaK* were bound with micromolar affinity. ATP-induced oligomerization of *FlaI* induced kinetic changes, stimulated fast-on, fast-off binding and lowered affinity (McMurry et al., 2015). Wang S. et al. (2010) illustrated that, under

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**Table 2**

KEGG pathway enrichment analyses.

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

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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.
chlorine oxidation, the flagella basal body operon \( fliBCDEFG \) and flagella biosynthesis factors such as \( flmA \) and \( flMB \) 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 \( \alpha \)-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 \( C\text{-CitB} \) family.** Cta sensor histidine kinase decreased (−1.16 log₂(FC)) to prevent extra citrate transport inside and Ctc (1.68 log₂(FC)) and Ctx (1.65 log₂(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 & Slalch, 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 \( fliDC \) 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 \( fliDC \) 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 Flagellar 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 & Tokumo, 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 & Tokumo, 2002). Gene \( cyw \), SEN 2261 (2Fe-2S ferredoxin), Fep, gene \( fhuD \), SEN 3272 (bacterioferritin-associated ferredoxin), gene \( sitABC \) were
unregulated. These phenomena confirmed that ATR S. Enteritidis went through an oxidation 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 log2(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 oxidation 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 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, and TtrC is an integral membrane protein containing a quinol oxidization 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 ppbC associated with TCA and gene ribB associated with production of FMN and FAD was up-regulated. Gene ulaDE and ulaG associated with 1-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 (dhpAB) 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 (phase 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 log2(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 oxidation 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 survivals. 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.

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Conflict of interest

Shuangfang Hu declares that she has no conflict of interest. Yi gang 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 she 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.

References
