Enhanced Virulence of Salmonella enterica serovar Enteritidis ATCC13076 under Acid Stress by Global Transcriptomics

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Abstract

Salmonella enterica serovar Enteritidis is a primary pathogen causing foodborne diseases and intestinal inflammatory responses. Acid tolerance response (ATR), as a strategy of adaption and resistance to acid stress, may contribute to enhanced virulence. In this study, there was a moderately acid adaption (pH 5.0) for *S*. Enteritidis cells prior to treatment with acid stress (pH 3.0). To figure out whether *S*. Enteritidis up-regulated the virulence or not, a global transcriptomic analysis was carried out by high-throughout RNA-sEq. The results showed 74 differentially expressed genes (DEGs) involved in virulence were identified after acid stress, among which, 62 DEGs were up-regulated and 12 DEGs were down-regulated. Afterwards, those virulence-linked DEGs were discussed and classified into four aspects based on the steps of infection, including flagellar functions, fimbrial adhesins, T3SS-mediated invasion and other virulent determinants. In conclusion, *S*. Enteritidis seemed to exhibit a trend of virulent genes towards high-expression under acid stress, revealing risks of *Salmonella* in acid-containing food. To our knowledge, there were few studies on comprehensively analyzing virulent genes expression changes of *Salmonella*, but it's novel to put forward pathogenicity as the highest priority under acid environment.

Introduction

Throughout the world, *Salmonella enterica* serovar Enteritidis and Typhimurium respectively ranking as the first and second places of the common serovars, are linked with outbreaks of human salmonellosis (Hendriksen et al. 2011). In spite of most studies focusing on *S*. Typhimurium, there are few on *S.* Enteritidis, which is responsible for some foodborne diseases and intestinal inflammatory responses. A survey about the worldwide distribution in human cases (Ekdahl et al. 2005), suggested *S*. Enteritidis is the most prevalent serovar in all but Africa, India and America. Besides, there have happened a series of outbreaks triggered by *S*. Enteritidis (Mandilara et al. 2017; Pavlova et al. 2018; Wright et al. 2015). Since researchers realized this serotype posed great risks to food industry and public health, a great many relevant reports have been published to figure out the mechanisms of the virulence.

The flagellum is a required multifunctional complex to enable the cells motility of swarming and swimming, and its functions involve attachment and adhesion, colonization, biofilm formation, as well as chemotaxis (Oguri et al. 2019). After approaching to the targets, *Salmonella* deploy a variety of adhesive structures in its surface at the initial step. In a review, Wagner and Hensel (2011) have systematically elucidated those structures including fimbriae, flagellum and other non-fimbrial adhesins, participating in specificity of adhesion to the host cells and cooperating with other virulence determinants. Gene clusters of fimbriae, including *fim*, *csg*, *pef* and *lpf* (Ledeboer et al. 2006), contributed directly to its functions to a certain extent. Besides, lipopolysaccharide (LPS) and translocon proteins in T3SS are a second form of adhesins (Wagner and Hensel 2011).

In terms of the pathogenicity of *Salmonella*, the genes located on pathogenicity islands were attached great importance to. With the progress of research on pathogens, type III secretion system (T3SS) was believed to exist in some bacteria as a sophisticated machine that could attack and invade eukaryotic cells to manipulate the host cells, including *Salmonella*. The invasion is achieved with a needle-shaped structure, a vital component of T3SS, to deliver virulent proteins termed "effectors" (Cascales 2017). It was confirmed that

Salmonella pathogenicity island 1 and 2 (SPI-1,2) encoded T3SS-1,2 that synthesized two types of effectors respectively. The effectors translocated by T3SS-1 are required for invasion into host cells and *Salmonella*-containing vacuole (SCV) biogenesis, such as SopA, SopB, SopD, SopE (Fabrega and Vila 2013). While T3SS-2 effectors are linked to SCV maturation, replication and achievement of infection inside host cells, including SseL, SifA, SopD2, SopE2 and PipB2 (McGhie et al. 2009). SopA, a HECT-type E3 ligase, plays a key role in the stimulation of inflammation when an animal is infected with *Salmonella* Typhimurium. (Kamanova et al. 2016). SopD was found to directly bind to the InvC ATPase (Boonyom et al. 2010). SopE is a guanidyl exchange factor (GEF) for Rac1 and Cdc42, secreted by the T3SS-1 upon host cell contact and promotes entry through triggering of actin-dependent ruffles (Vonaesch et al. 2014). SseL is retained predominantly in the cytoplasm of infected cells following translocation by the T3SS (Coombes et al. 2007). SifA is related to localize to *Salmonella*-induced filaments (SIFs) and enables continuous fusion of host vesicles to SCV membranes (Knuff and Finlay 2017).

A series of stresses have been verified to render *Salmonella* to adjust its pathogenicity as survival strategies. For instance, Shah et al. (2012) performed an experiment in which acid and oxidative stresses impaired intestinal colonization and systemic dissemination in orally inoculated chickens. Meanwhile, the study showed the stress-resistance significantly changed the expression of some virulence genes. Especially, *Salmonella* have already acquired the ability to survive acid environment such as the gastrointestinal tract of their hosts. Acid tolerance response (ATR) depends on various strains (Lianou et al. 2017), types acid and its acidity (Alvarez-Ordonez et al. 2009), duration and so on. The virulent aspect of ATR remains ambiguous over *S.* Enteritidis, posing a great threat to the safety of acidic food and beverages. The molecular understanding of *S.* Enteritidis pathogenicity under acid stress is necessary for further insight and food industry.

Results

1 Data processing and DEGs analysis

After filtration and processing, the results showed the basic data of RNA sequencing, which suggested they are qualified and credible for following analysis and discussion. According to the threshold of log2(FC) fold-change ≥ 1, FDR < 0.05, 554 DEGs were identified under acid stress. And then, 74 DEGs involved in virulence were classified and analyzed referring to the description of genes, Fabrega and Vila (2013), Wagner and Hensel (2011), as well as using the website (http://www.mgc.ac.cn/cgi-bin/VFs/genus.cgi?Genus=Salmonella). Among those selected DEGs, 62 DEGs were up-regulated and 12 DEGs were down-regulated, shown in the volcano plots diagram (Fig. 1).

2 GO analysis classification of DEGs

GO analysis (Quinteiro-Filho et al. 2012) were employed to classify the functions of identified DEGs. GO analysis results showed the percentage (number) of virulence-associated DEGs in various parts of cellular component, molecular function and biological process according to GO database (Gelli et al. 2017). Within cellular component, a big part of DEGs which were related to cell projection and bacterial-type flagellum

accounted for the first two largest clusters. And in the category of biological process, a high percentage of DEGs existed in locomotion and cell process, as well as biological adhesion.

3 KEGG analysis classification of DEGs

In order to understand the virulent responses of acid mediated pathways in ATR. KEGG pathway enrichment analysis was also conducted. According to results of analysis, pathways including flagellar assembly, bacterial secretion system, bacterial chemotaxis, lipopolysaccharide biosynthesis and biofilm formation were in significant up-regulation under such an acid stress.

4 Validation of DEGs using qRT-PCR

In order to testify if the results of RNA-seq was in accord with the qPCR, some DEGs were analyzed through qPCR. Nine genes that include 7 up-regulated and 2 down-regulated genes were selected for the qRT-PCR analysis and used to validate the RNA-seq data from the preliminary experiment. It was shown in Fig. 2 that qRT-PCR data correlated well with the RNA-seq data (R^2 = 0.9117). Totally, the qRT-PCR data were paralleled to those of RNA-seq aiming at these genes, in spite that the specific values of fold-change were different.

Discussion

As a consequence of virulence potentials and the high incidence of *Salmonella*-specific infections, we put forward virulence of *S.* Enteritidis to be the highest priority in this study. Generally, the infection caused by *Salmonella* should be attributed to several virulent structures, such as flagellum, fimbriae, T3SS-mediated invasion, lipopolysaccharide (LPS) and regulators. On the other hand, different structures are responsible for different processes and functions as follows.

1 Up-regulated flagellar genes

In *Salmonella*, flagellum plays a crucial role in infection, especially in the initial phase, such as colonization and attachment to the intestinal epithelium (Barbosa et al. 2017; Salehi et al. 2017). The two major functions of flagellum include motility and chemotaxis. The synthesis and function of the flagellar and chemotaxis system requires the expression of more than 50 genes or at least 17 operons (*flh*, *flg*, *fli*, *flj*, *mot*, *che*, *tar*, *tsr*, and *aer*) that constitute the large and coordinately regulated flagellar regulon (Chilcott and Hughes 2000). It is demonstrated in Fig. 3 that flagellum consists of three parts, filament, hook, and basal body. Main component proteins of filament and hook are FliCD and FlgE respectively, and the latter one is regulated by FliK and FlgD. While *flh* and *flj* didn't show a significant up-regulation. In our study, *fliC* was up-regulated by 2.13 and *fliD* by 1.48 $\log_2$(FC), as well as *flgE* by 1.05. Crawford et al. (2010) reported that FliC can mediate binding to cholesterol and initiating biofilm formation, suggesting flagella enhanced bacterial adhesion. The center part of basal body is type III injectisome, a sophisticated nanomachine similar to T3SS (Erhardt et al. 2010). FliI (+ 1.32 $\log_2$(FC)) was identified as an ATPase along with FliH (+ 1.24 $\log_2$(FC)), both involved in the apparatus of type III injectisome (Minamino 2014). Apart from the structure protein described above, proximal rod (FlgB, FlgC and FlgF), MS ring (FliF) along with C ring (FliG) distinctly had an up-regulation by 1.09 ~ 1.44 $\log_2$(FC).

Chevance and Hughes (2008) have elaborated on the mechanism of flagellar assembly: a three-tier hierarchy regulation. According to our data, in spite of the first class genes (*flhDC* operon) not showing different expressions, the high-expressed FliA by 1.19 and FliZ by 1.13 $\log_2$(FC), both of which belonged to class 2. And FliA was identified as the sigma factor (σ^{28}) of flagellar genes, while FliZ as the expression activator to regulate its own class genes (Tanabe et al. 2011). Actually, class 2 proteins couldn't express in absence of $FlhD_4C_2$ revealing a fact that *flhDC* operon had finished its work in earlier stage as shown in Fig. 3 (Barker et al. 2014). In addition, *flgANM* and *fliT* were all significantly up-regulated coincidently in varying levels ranging from 1.02 to 1.50 $\log_2$(FC). Prior to the completion of class 2 assembly (Fig. 3), the anti-σ^{28} factor FlgM regulated the expression of late substrate genes under regulation of class 3 promotor and hook-associated genes (*flgKL and fliD*) (Erhardt et al. 2010). Moreover, genes of chemotaxis (*cheYZ*, *tsr* and *aer*) were also respectively up-regulated by 1.24, 1.19 1.37 and 1.12 $\log_2$(FC) under acid stress. Whereas the motility genes (*motAB*) did not appear a significant up-regulation as expectation. Obviously, what are listed above offers the amplest evidence that flagella functioned better in such extreme situation.

2 Fimbriae-linked genes were slightly up-regulated

Previous to *Salmonella*'s invasion into host cells, fimbriae is a required and decisive structure to adhere to cell membrane. As shown in Table 2, *S*. Enteritidis up-regulated majority of genes involved in fimbrial assembly, transporter, structure, regulator and chaperone/usher protein. Fimbrial adhesins are produced in following three pathways referring to Wagner and Hensel (2011): chaperone-usher pathway, the extracellular nucleation pathway and type IV pili. *fimD* and *fimI*, members of type I fimbriae (SEF21) family, generally appeared a down-regulation by 1.06 and 1.54 $\log_2$(FC), however, *fimF* was up-regulated by 1.36 $\log_2$(FC). Another type of fimbriae -SEF14- existed specifically in *S*. Enteritidis. It turned out to be three genes (*sefABC*) in SEF14 operon. SEF14 fimbriae contributes to SE adherence with mouse epithelial cells but not to human HEp-2, Caco-2, INT-407, or HeLa cells (Quan et al. 2019). SefA, a novel fimbrin, owns the ability to enhance *S*. Enteritidis adhesion to epithelial cells and survival in macrophages and results in *S*. Enteritidis virulence in mice (Zhu et al. 2013). SefB and SefC shared homology with *E. coli* fimbrial chaperone and outer membrane proteins (Clouthier et al. 1993). From our results, *sefABC* was up-regulated by 1.54, 1.45 and 1.90 $\log_2$(FC), respectively. In addition, type IV pili genes seemed to be regulated during acid stress, *hofC* showing a down-regulation by 1.46 and *pilCN* by 1.67 and 1.00 except for SEN1977. As far as long polar fimbriae (Lpf) was concerned, it was firstly proposed to role in the adhesion of *S*. Typhimurium to cells of the Peyer's patches (Bäumler et al. 1996). In our study, *lpfA* acting as a precursor of long polar fimbrial protein A up-regulated by 2.09 $\log_2$(FC) than unstressed cells. Such abundance of expression suggested the cells were likely to up-regulate the subsequent genes (*lpfCD*) in the next steps. On the other hand, *Salmonella* can specifically assemble curli fiber or thin aggregation fimbriae (Tafi) via the nucleation-participation pathway like *E. coli*. Curli fimbriae relates to adhesion, induction of proinflammatory response and biofim formation (Fabrega and Vila 2013). And CsgBA (subunits) and CsgDEFG are required for curli biogenesis, these two divergently transcribed operons are the homologues in *E. coli* (Römling et al. 1998). In a recent study (Newman et al. 2018), it was unraveled that regulator CsgD mediated stationary phase counter-silencing of *csgBA* in *S*. Typhimurium. Under acid stress, *S*. Enteritidis also up-regulated *csgD* by 1.84 and *csgC* by 1.71 $\log_2$(FC) in site of insignificance (FDR $\geq$ 0.05), not shown in Table 2, as well as other protein with slightly up-regulation. Moreover, up-regulations of *yehD* encoding a fimbrial subunit protein and *virF* encoding a fimbrial operon positive regulatory protein reflected an

active state of fimbriae. In total, roles of fimbriae in adhesion turned out to be a reasonable enhancement even though *S*. Enteritidis cells partly down-regulated type I fimbriae and type IV pilin.

3 *S*. Enteritidis up-regulated genes for invasion and infection

T3SS-1 was confirmed to enable *Salmonella* to invade into host epithelial cells and establishing infection in the gastrointestinal tract. T3SS-1 would not function completely until DsbA arrived its destination and activated the apparatus, which is required for translocation and secretion of effectors (Fabrega and Vila 2013). After treated with acid stress, *S*. Enteritidis slightly up-regulated *dsbA* by 0.79 $\log_2$(FC). Initiation of actin cytoskeletal remodeling and induction of a proinflammatory response are attributed mainly to the effectors SopE, SopE2 that function as a guanidine exchange factor (GEF) for host cellular Rho GTPases (Hardt et al. 1998). Despite of the differences of their structures, these two proteins function in invasion into host cell identically. Our data shows *S*. Enteritidis up-regulated DsbA by 0.79, SopE and SopE2 by 1.48 $\log_2$(FC). Two additional key SPI-1 effectors, SopA and SopD also contributing to enteropathogenesis. In this study, SopA was down-regulated by 1.13 $\log_2$(FC), while SopD was up-regulated by 2.04 $\log_2$(FC). Kamanova et al. (2016) concluded that *sopA* encodes a HECT-type E3 ligase, contributing to the stimulation of inflammation when an animal is infected with *S*. Typhimurium. It was reported SopD directly bound to the InvC ATPase and was found in the host-cell cytosol not only during the early stages of infection but also later in the SCV (Boonyom et al. 2010; Brumell et al. 2003). Moreover, SopD and SopB play role cooperatively in enhancing membrane fission and to promote micropinocytosis during *S*. Typhimurium invasion (Bakowski et al. 2007). In addition, T3SS encoded by SPI-2 generally functions and translocates effectors that participate in SCV maturation and establishment of infection within host cells. As shown in Table 2, there is an up-regulation of 1.19 $\log_2$(FC) for SseL. Such a T3SS-2 effector protein was reported to retain predominantly in the cytoplasm of infected cells following translocation by the T3SS (Coombes et al. 2007). PipB2 and SifB as another two translocated effectors of T3SS-2 were respectively up-regulated by 1.57 and 1.22 $\log_2$(FC). And it has been proven that PipB2 and SifB can localize to SIF and SCV membranes and result in subsequent infection (Freeman et al. 2003; Knuff and Finlay 2017). Apart from the effectors, SsaQ (type III secretion system protein), SseC (translocation machinery component), RcsC (secretion system regulator: Sensor component) and SEN3371 (pathogenicity island protein) were also significantly up-regulated by 1.01 ~ 1.43 $\log_2$(FC).

Surprisingly, *inv*, *hilA*, *spt*, *spa*, *sip*, *iag*, *iac* and *sic* that had a close connection with colonization and invasion of *Salmonella* referring to earlier studies (Bajaj et al. 1996; Boyd et al. 1997; Phoebe Lostroh and Lee 2001; Rahn et al. 1992), turned out to be no expression in either unstressed or stressed cells in our results. The unexpected phenomenon could attribute to the hierarchy of regulation of invasion. Gene regulation plays an extremely important role in the efficacy of the pathogenesis of *Salmonella* in order to coordinate all the virulence traits (Fabrega and Vila 2013). As shown in Fig. 4, this regulation is under a temporal hierarchy in which virulence elements need to be progressively expressed. In our study, *S*. Enteritidis mildly up-regulated HilD by 0.99 $\log_2$(FC), which was regarded as the most important activator of HilA *in vitro* and at the upper class of the hierarchy. Gene *hilD* was activated by FliZ and Fis, but repressed by CsrA, all the three type of regulator was up-regulated by 1.13 ~ 1.29. And the presence of Hha (Nucleoid proteins) with an up-regulation of 1.39 $\log_2$(FC) absolutely repressed *hilA*, contributing to repression of *inv*/*spa*, *prg*/*org*, *sic*/*sip* and some effectors. Furthermore, physiology of *S*. Enteritidis should be considered. If not in a period of invasion, it is not

an economic alternative to maintain a high level of expression for these proteins. As a result, regulators such as DsbA and HilD made a greater contribution to the subsequent invasion, let alone the various effectors encoded by SPI-1 and SPI-2.

4 Other pathogenicity determinants were up-regulated

4.1 LPS

LPS, located in the outer layer of membrane, not only functions as a defender of cells, but is also identified as a key determinant of virulence involved in colonization (Nevola et al. 1985), invasion and self-protection from bacteria being killed by macrophages (Kong et al. 2011). It consists of three components, including lipid A, core oligosaccharide (C-OS), and O-antigen polysaccharide. The assembly system of C-OS A *rfaL* mutant of *S.* Typhimurium, which lacks the entire O-antigen (O-Ag), was virtually avirulent in a *Galleria mellonella* infection model (Bender et al. 2013). According to our data (Table 2), not only was rfaL up-regulated by 1.37 $\log_2$(FC). Besides, Kong et al. (2011) also elucidated *rfaGIHJL*, *rfbP* and *rfc* encoded a series of expressions that were concerned with LPS's virulent aspect. The results showed that genes in Rfa, Rfb and Rfc families were up-regulated by 1.17 ~ 2.23 $\log_2$(FC) except for RfaF's down-regulation by 1.20 $\log_2$(FC).

4.2 Regulators

Importantly, CsrA, a global post-transcriptional regulator controlling various properties related to metabolism and virulence, had a significant high-expression by 1.23 $\log_2$(FC). For instance, CsrA was proved to positively regulate FlhC and FlhD (Lawhon et al. 2003), both the subunits of the primary regulator in flagella expression, which didn't show an obvious up-regulation according to our data. Additionally, Sterzenbach et al. (2013) figured out that CsrA is a positive regulator of PefA expression in *S.* Typhimurium by binding a GGA motif in the 50-UTR of the *pefACDEF* transcript. Meanwhile, capability of regulating HilD leaves CsrA involved in invasion, especially in the expression of the T3SS part (Fortune et al. 2006).

In this study, *marA* and *soxS* was up-regulated by 2.37 and 1.27 $\log_2$(FC), respectively. MarA was proved to effect the invasion into host cells for *S.* Choleraesuis, when exposed to an environment of acid and bile salts (Lee et al. 2015). In *E. coli*, MarA and SoxS were both described as members of AraC family to regulate a great many proteins, when adapting to unfavorable conditions, including acid stress (Duval and Lister 2013). Another study also implicated that SoxS controlling expression of distinct genes acted as a key factor in persistence of *E. coli* in murine pyelonephritis (Casaz et al. 2006).

Conclusion

Altogether, by using global transcriptomic, we surprisingly found that *S.* Enteritidis enhanced its virulence after exposed to such an acid environment. We analyzed and divided results into four essential virulence-associated aspects, including flagellar functions, fimbriae-mediated adhesion, invasion of T3SS, virulent roles of LPS and some regulators. Each part showing an up-regulation contributed jointly and strongly to our conclusion that *Salmonella* Enteritidis had evolved the ability to cope with low pH environment such as the gastrointestinal tract of their hosts during infection. This study paid attention to a threat of *Salmonella* existing in acid food, as

an inspiration for the food industry. Furthermore, it's valuable to figure out the mechanisms of virulent aspects in *Salmonella*.

Material And Methods

1 Bacterial strain and gastric fluid simulation

The strain used in this study was *Salmonella* enterica subsp. enterica serovar Enteritidis ATCC13076, stocked at -80°C in Luria-Bertani (LB) with 30% glycerol. Initially, it was activated by streaking onto LB plate with incubation at 37°C for 24 h before each experiment. Then the exponential phase cells (OD600 ≈ 1.0) were centrifuged at 5000×g for 3 min and washed twice with sterilized phosphate buffer. Subsequently, the washed culture was dissolved in LB broth at a concentration of approximately 10^6 CFU/mL (6 Log 10 culture).

Under acid stress, *S*. Enteritidis was pretreated with acid adaption as follows. Briefly, during acid adaption process, 6 Log10 culture was collected in the LB broth (pH 5.5) for 1 h. And immediately, the broth was changed to pH 3.0 with HCl (1 M) or incubated for another hour. For the control, treatment in LB broth (pH 7.0) for 2 h was conducted. The temperature throughout the duration of above experiments was maintained at 37°C.

2 RNA isolation and library construction

According to the instruction from manufacturer, the total RNA of *S*. Enteritidis in each sample was extracted by using the Trizol Kit (Promega, USA). There existed some DNA left in the total RNA to be degraded by RNase-free DNase I (Takara Bio, Japan) for 30 min at 37°C. Results of RNase free agarose gel electrophoresis and an Agilent 2100 Bio-analyzer (Agilent Technologies, Santa Clara, CA) can testify if RNA isolation was of good quality. Afterwards, Poly (A) mRNA isolated by oligo-dT beads (Qiagen) was fragmented in the lysis buffer.

According to the fragmented mRNA, there generated the first-strand cDNA through random hexamer-primed reverse transcription. Subsequently, the synthesis of the second-strand cDNA took place in presence of RNase H and DNA polymerase I (Chen et al. 2016). A QIA quick PCR extraction kit was used to enable the purification of cDNA fragments. These purified fragments were subjected to EB buffer for end reparation poly (A) addition and ligated to sequencing adapters. At last, extraction, purification and enrichment of the ligated cDNA fragments were conducted by PCR aiming to construct cDNA library after agarose gel electrophoresis.

3 Sequencing and reads alignment

It was performed on the Illumina sequencing platform (Illumina HiSeq™ 2000) to sequence the cDNA library with the pairedend technology applied (Gene Denovo Co. Guangzhou, China). A Perl program was employed to remove low quality sequences that refer to more than half of entire bases with quality lower than 20 or reads with more than 5% N bases (N means bases unknown) and adaptor sequences (Chen et al. 2016). Short sequences alignment was conducted with SOAPaligner/soap2 paralleled to a previous research. These sequencing reads were subsequently mapped to reference sequence by the SOAPaligner/soap2 (Li et al. 2009). The expression statistics and visualization throughout this study was accomplished with the technical assistance of R package (http://www.r-project.org/).

4 Differentially expressed genes (DEGs) and function enrichment analyses

The Bioconductor package edgeR (Robinson, McCarthy, & Smyth, 2010) was employed in identifying of differential expression genes. As the false discovery rate (FDR) was to examine and filter out the genes without significance in differential expression, we applied a p value, a threshold of the FDR ≤ 0.05 and $|\log_2 Ratio| \geq 1$ according to Chen et al. (2016).

Referring to a previous study (Zhang et al. 2013) with minor modification, the DEGs were then subjected to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses. Once a P-value was not more than 0.05, we considered that both two methods have achieved a significant enrichment.

5 Quantitative real-time PCR (qRT-PCR) validations

It was 7500 Fast Real-Time PCR System (Applied Biosystem, Foster, USA) that was employed to justify the uniformity of gene expression between RNA-seq and qRT-PCR, with 9 DEGs selected. We regarded the 16 S RNA gene of *S*. Enteritidis as the internal control gene. In accordance with instruction manual, total RNA extraction was conducted with Trizol reagent, and the extracted RNA was instantly utilized in cDNA synthesis by using reverse transcriptase. Quantitation of each transcript was performed with total RNA in duplicate and the starting materials and each qPCR were conducted in triplicate.

Table 1 outlined the primer pairs used in qPCR analysis and some sequences for detection of *S*. Enteritidis derived from those of references with modifications.

Declarations

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Conflicts of interest

Hong Bai, Donggen Zhou, Shuangfang Hu, Xiaowei Zhang, Qijun Liu, Xinglong Xiao, Yigang Yu and Xiaofeng Li declare that they have no conflict of interest.

Availability of data and material

Not applicable.

Code availability

Not applicable.

Authors' contributions

Hong Bai and Donggen Zhou had substantial contributions to the experiments, as well as the processing and analysis of data in this study; Shuangfang Hu, Xiaowei Zhang, Qijun Liu and Xinglong Xiao helped with the analysis and interpretation of transcriptional data of the work. And finally, Yigang Yu and Xiaofeng Li revised the final draft of this article critically and made it acceptable for publication.

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Tables

Table 1
Primer pairs used for qRT-PCR validation.

Gene name	Gene annotation	Primer pair	Reference
16S rRNA		5’-CCTCAGCACATTGACGTTAC-3’ 5’-TTCCTCCAGATCTCTACGCA-3’	(Eaves et al., 2004)
sefC	outer membrane fimbrial usher protein	F,5’-GGCAGGTCCAAAACTATACA-3’ R,5’-GCGATAACGAAACACCATTT-3’	(Choudhury et al., 2016)
sopE	type III secretion system, secreted effector protein SopE	F,5’-ACACACTTTCCACGAGGAAGCG-3’ R,5’-GGATGCCTTCTGATGTTGACTGG-3’	(El-Sharkawy et al., 2017)
sopA	secreted protein SopA	F,5’-ATCGATGGACTGTCATTGAATAATCAGC-3’ R,5’-ATCGATGGTTGAGGCTGGACTAC-3’	(Raffatellu et al., 2005)
rfbS	paratose synthase	F,5'-ACATACTGTGATTGGCTTAG-3' R,5'-CATTGGCTCTTTCTTTGA – 3'	(Ren et al., 2016)
sseL	conserved hypothetical protein; deubiquitinase	F,5’-AGGAAACAGAGCAAAATGAA-3’ R,5’-TAAATTCTTCGCAGAGCATC-3’	(Liu et al., 2011)
fimI	major pilin protein	F,5’-CCTTTCTCCATCGTCCTGAA-3’)-3’ R,5’-TGGTGTTATCTGCCTGACC-3’) -3’	(Jothikumar et al., 2003)
pipB2	exported protein	F,5’-TCTTCCTGGTGAGAATAACG-3’ R,5’-TTCGCATCTGAATAGTAAAGC-3’	(Yin et al., 2017)
oatA	lipopolysaccharide modification acyltransferase	F,5’-GTCAGCTTCCGGCGTGGCCGCGGATAACAA GAGAGAGTGTAGGCTGGAGCTGCTTC-3’ R,5’-AGATGCCTTCATCGAGTAGTTGGATATGTC CAGCT ACATATGAATATCCTCCTTAG-3’	(VanDrisse and Escalante-Semerena, 2018)
csrA	carbon storage regulator	F,5’-CTGGACTGCTGGGATTTTTC-3’ R,5’-CATGATTGGCGATGAGGTC-3’	(Mizusaki et al., 2008)

Table 2
Classification of differentially expressed genes (DEGs) associated with virulence according to functions and structures when *S.* Enteritidis was coping with acid stress.

Classification	Gene ID	Name	Fold change (log_2FC)	P-value	FDR	Description
flagella	SEN1037	*fliI*	1.32	1.47E-25	1.83E-24	Flagellum-specific ATP synthase FliI
	SEN1038	*fliH*	1.24	4.52E-24	5.22E-23	flagellar assembly protein H
	SEN1039	*fliG*	1.44	4.59E-52	1.32E-50	flagellar motor switch protein G
	SEN1040	*fliF*	1.19	2.64E-38	5.20E-37	flagellar MS-ring protein
	SEN1046	*fliT*	1.30	2.71E-18	2.46E-17	flagellar biosynthesis protein FliT
	SEN1048	*fliD*	1.48	6.87E-258	1.50E-255	flagellar capping protein
	SEN1049	*fliC*	2.13	0	0	flagellin
	SEN1051	*fliA*	1.19	8.45E-40	1.71E-38	flagellar biosynthesis sigma factor, partial
	SEN1052	*fliZ*	1.13	1.10E-32	1.81E-31	flagella biosynthesis protein FliZ
	SEN1087	*cheY*	1.24	3.05E-22	3.26E-21	chemotaxis regulatory protein CheY
	SEN1088	*cheZ*	1.19	1.65E-44	3.96E-43	chemotaxis regulator CheZ
	SEN1870	*flgF*	1.40	7.77E-26	9.80E-25	flagellar basal body rod protein FlgF
	SEN1871	*flgE*	1.05	1.75E-51	4.96E-50	flagellar hook protein FlgE
	SEN1873	*flgC*	1.37	9.56E-11	5.46E-10	flagellar basal body rod protein FlgC
	SEN1874	*flgB*	1.09	2.10E-08	1.01E-07	flagellar basal body rod protein FlgB
	SEN1875	*flgA*	1.43	4.84E-23	5.36E-22	flagellar basal body P-ring biosynthesis protein FlgA
	SEN1876	*flgM*	1.02	1.61E-10	9.05E-10	anti-sigma-28 factor FlgM
	SEN1877	*flgN*	1.50	1.35E-38	2.68E-37	flagellar synthesis protein FlgN

Classification	Gene ID	Name	Fold change (log_2FC)	P-value	FDR	Description
	SEN3059	*aer*	1.12	1.96E-14	1.42E-13	aerotaxis receptor
	SEN4145	*bsmA*	-2.21	7.01E-143	6.31E-141	biofilm stress and motility protein A
	SEN4298	*tsr*	1.37	8.25E-162	8.99E-160	methyl-accepting chemotaxis protein I
fimbria	SEN0146	*hofC*	-1.46	0.007687	0.017094	type IV pilin biogenesis protein
	SEN0525	*fimI*	-1.54	0.005139	0.011935	major pilin protein
	SEN0527	*fimD*	-1.06	6.39E-07	2.62E-06	outer membrane usher protein SfmD
	SEN0529	*fimF*	1.36	0.011403	0.024544	fimbrial protein
	SEN1977	-	1.23	5.29E-13	3.51E-12	putative type IV prepilin
	SEN2145B	*yehD*	2.02	6.04E-12	3.78E-11	fimbrial subunit protein
	SEN2875	*pagC*	3.13	7.28E-37	1.36E-35	outer membrane protein
	SEN3463	*lpfA*	2.09	0.002614	0.006374	long polar fimbrial protein A precursor
	SEN4247	*sefA*	1.54	5.32E-91	2.72E-89	fimbrial protein
	SEN4249	*sefC*	1.90	3.12E-06	1.18E-05	outer membrane fimbrial usher protein
	SEN4251	*virF*	1.29	0.000154	0.00046	fimbrial operon positive regulatory protein
	SEN4350	*lpfB*	1.37	0.005139	0.011935	fimbrial assembly chaperone SthB
invasion	SEN0454	*hha*	1.39	1.06E-09	5.60E-09	hemolysin expression-modulating protein
	SEN1155	*sopE*	1.48	0.010733	0.023208	type III secretion system, secreted effector protein SopE
	SEN1454	*sifB*	1.22	0.011591	0.024871	secreted effector protein
	SEN1627	*ssaQ*	1.43	7.78E-05	0.000243	type III secretion system protein
	SEN1645	*sseC*	1.19	0.006667	0.014997	translocation machinery component

Classification	Gene ID	Name	Fold change (log_2FC)	P-value	FDR	Description
	SEN1653	*rcsC*	1.01	5.52E-08	2.52E-07	secretion system regulator: Sensor component
	SEN2065	*sopA*	-1.13	2.86E-06	1.09E-05	secreted protein SopA
	SEN2269	*sseL*	1.19	2.45E-05	8.25E-05	conserved hypothetical protein; deubiquitinase
	SEN2624	*pipB2*	1.57	2.11E-13	1.44E-12	exported protein
	SEN2784	*sopD*	2.04	1.57E-05	5.40E-05	Sop effector protein SopD
	SEN3219	*fis*	1.29	4.63E-30	6.85E-29	Fis family transcriptional regulator
	SEN3371	-	1.12	1.94E-41	4.19E-40	pathogenicity island protein
lipopolysaccharide	SEN1893	*lpxL*	-1.03	2.00E-13	1.37E-12	lipid A biosynthesis lauroyl acyltransferase
	SEN2082	*rfbK*	1.17	1.59E-13	1.10E-12	phosphomannomutase
	SEN2085	*rfbU*	1.43	9.82E-13	6.42E-12	mannosyl transferase
	SEN2085C	*rfbE*	1.67	4.80E-44	1.14E-42	CDP-paratose 2-epimerase
	SEN2085D	*rfbS*	2.30	2.23E-29	3.21E-28	paratose synthase
	SEN2378	*oatA*	1.98	0.000514	0.001401	lipopolysaccharide modification acyltransferase
	SEN2387	*lpxP*	1.05	1.36E-08	6.64E-08	lipid A biosynthesis palmitoleoyl acyltransferase
	SEN3533	*rfaF*	-1.20	1.78E-31	2.80E-30	ADP-heptose:LPS heptosyltransferase II
	SEN3535	*rfaL*	1.37	4.24E-48	1.14E-46	O-antigen ligase
	SEN3539	*rfaJ*	1.78	2.36E-20	2.33E-19	lipopolysaccharide 1,2-glucosyltransferase
Regulators	SEN1532	*marA*	2.37	4.05E-36	7.32E-35	DNA-binding transcriptional activator MarA
	SEN2667	*csrA*	1.23	6.61E-14	4.66E-13	carbon storage regulator

Classification	Gene ID	Name	Fold change (log_2FC)	P-value	FDR	Description
	SEN4035	*soxS*	1.27	7.69E-06	2.76E-05	DNA-binding transcriptional regulator SoxS

Figures

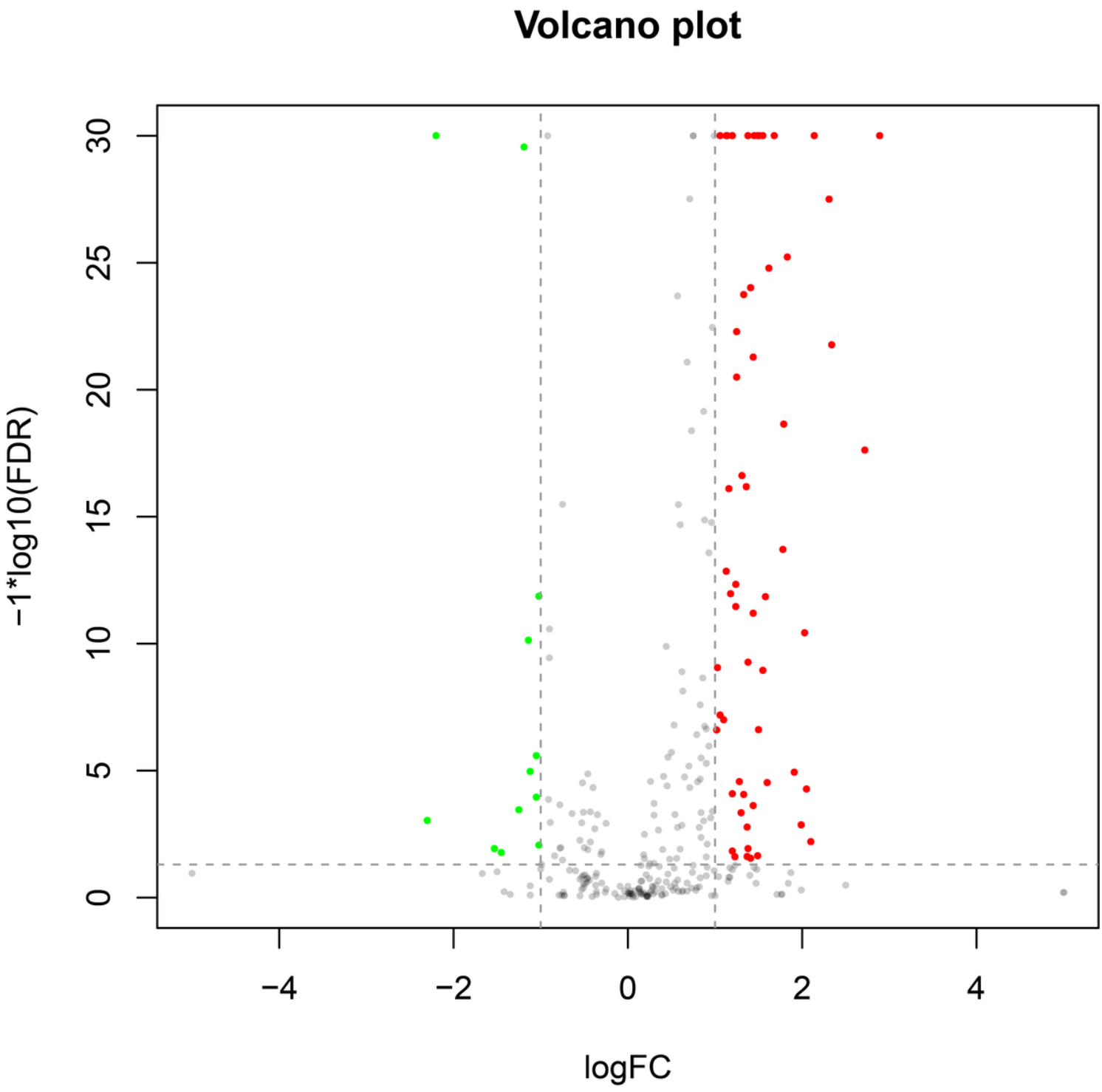

Figure 1

Volcano plots diagram of DEGs associated with virulence between acid stressed and unstressed S. Enteritidis. Red spots in the right part: up-regulated genes; green spots in the left part; down-regulated genes; black spots in the middle part: genes with insignificant changes between the stressed and unstressed.

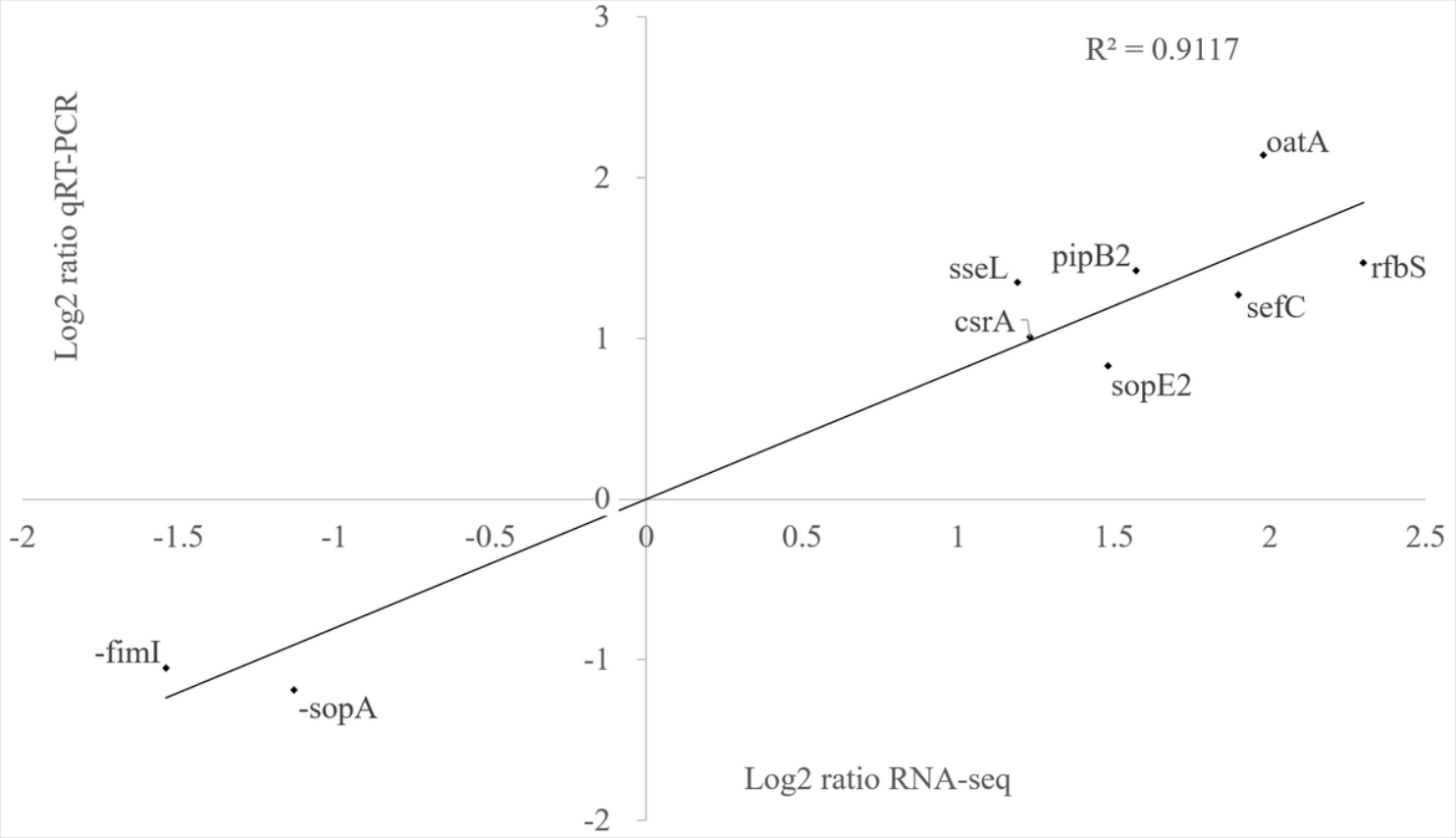

Figure 2

qRT-PCR validation. X: the log2 fold change according to RNA-seq; Y: the log2 fold change according to qRT-PCR.

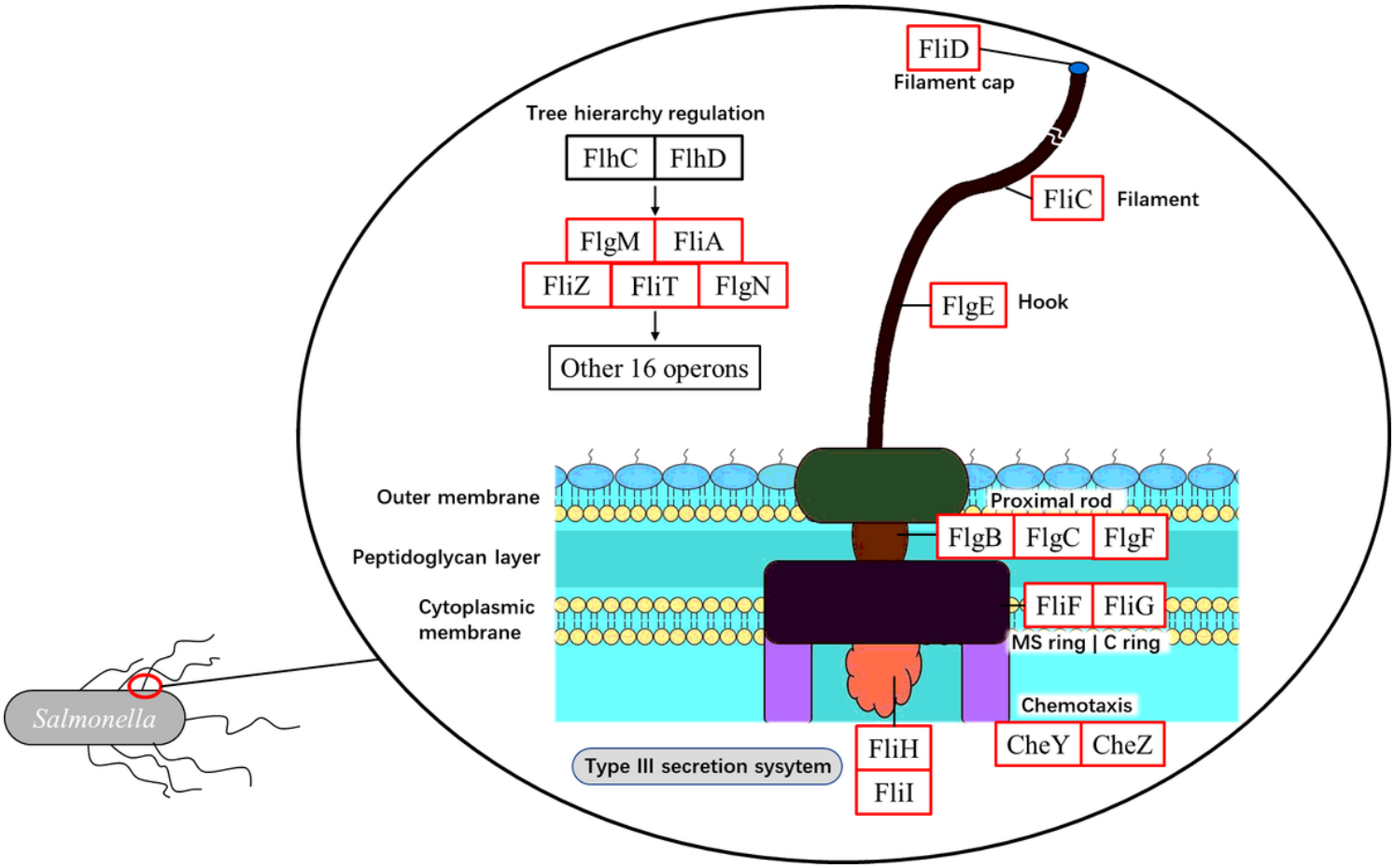

Figure 3

Flagellar assembly and chemotaxis were up-regulated under under acid stress in S. Enteritidis. Red boxes: up-regulated genes; black boxes: genes with insignificant changes between the stressed and unstressed.

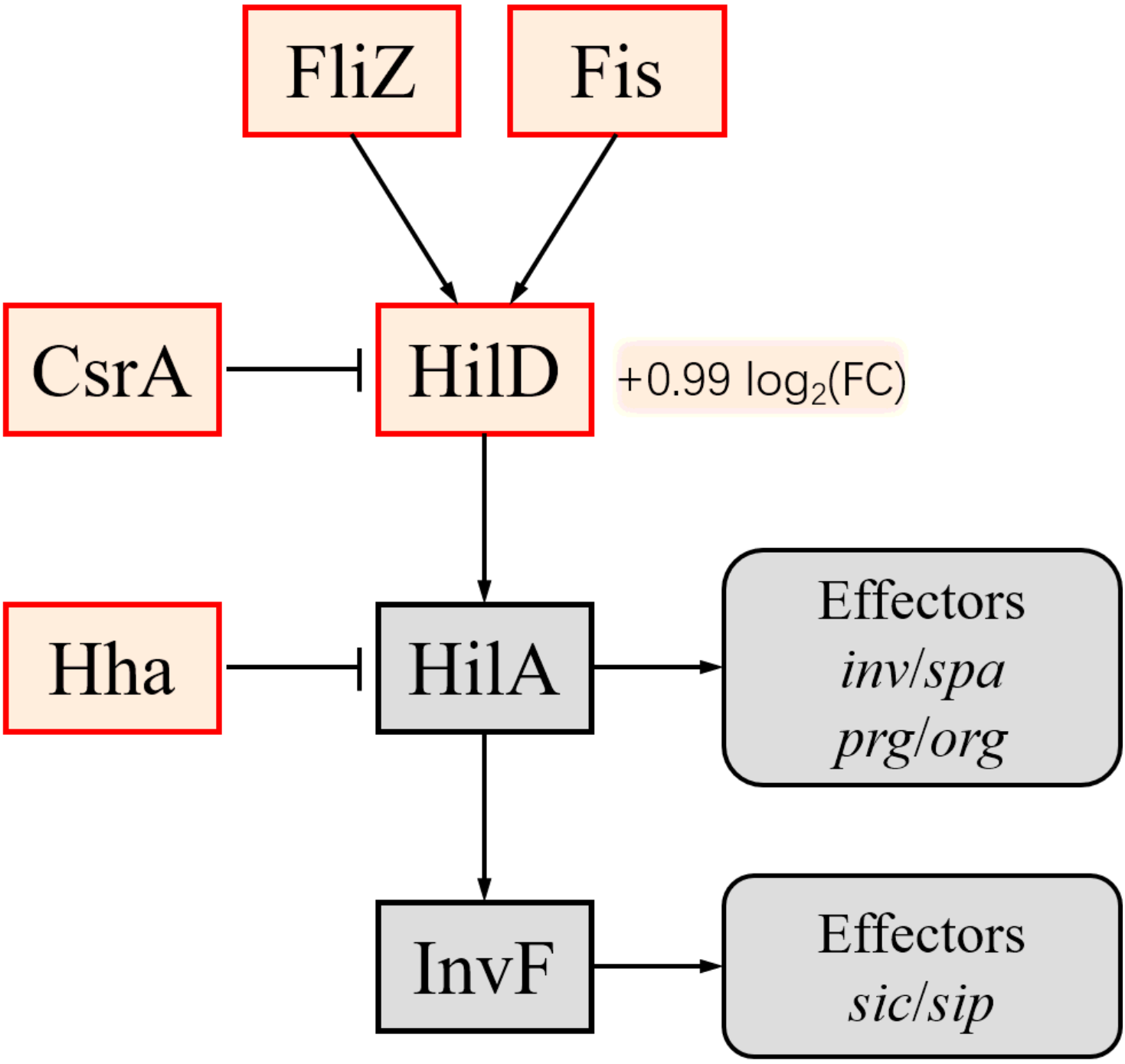

Figure 4

Model proposing the possible mechanism of hierarchy regulation of genes involved in invasion under acid stress condition. Common arrows: activation regulation; blunt-end arrows: repression regulation; Red boxes: up-regulated genes; black boxes: genes with insignificant changes between the stressed and unstressed (Fabrega and Vila 2013)