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Quantitative proteomics reveals the crucial role of YbgC for *Salmonella enterica* serovar Enteritidis survival in egg white

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ABSTRACT

Salmonella enterica serovar Enteritidis (*S.* Enteritidis) is a food-borne bacterial pathogen that can cause human salmonellosis predominately by contamination of eggs and egg products. However, its survival mechanisms in egg white are not fully understood, especially from a proteomic point of view. In this study, the proteomic profiles of *S.* Enteritidis in Luria-Bertani (LB) broth containing 50% and 80% egg white, and in whole egg white were compared with the profile in LB broth using iTRAQ technology to identify key proteins that were involved in *S.* Enteritidis survival in egg white. It was found that there were 303, 284 and 273 differentially expressed proteins in *S.* Enteritidis after 6 h exposure to whole, 80% and 50% egg white, respectively. Most of up-regulated proteins were primarily associated with iron acquisition, cofactor and amino acid biosynthesis, transporter, regulation and stress responses, whereas down-regulated proteins were mainly involved in energy metabolism, virulence as well as motility and chemotaxis. Three stress response-related proteins (YbgC, TolQ, TolA) of the *tol-pal* system responsible for maintaining cell membrane stability of Gram-negative bacteria were up-regulated in *S.* Enteritidis in response to whole egg white. Interestingly, deletion of *ybgC* resulted in a decreased resistance of *S.* Enteritidis to egg white. Compared with the wild type and complementary strains, a 3-log population reduction was observed in △*ybgC* mutant strain after incubation in whole egg white for 24 h. Cellular morphology of △*ybgC* mutant strain was altered from rods to spheres along with cell lysis in whole egg white. Furthermore, deletion of *ybgC* decreased the expression of *tol-pal* system-related genes (*tolR*, *tolA*). Collectively, these proteomic and mutagenic analysis reveal that YbgC is essential for *S.* Enteritidis survival in egg white.

1. Introduction

Out of the 2610 serotypes, *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) has been a continuous worldwide threat to public health over the last three decades because of the ability of this serotype to contaminate eggs (Braden, 2006; CDC, 2010; EFSA, 2012; EFSA and ECDC, 2015). Although *Salmonella* Typhimurium (*S.* Typhimurium) has also been shown to infect human through cracked eggs, *S.* Enteritidis is the only bacterium that routinely causes human infection *via* intact eggs (Kang et al., 2006). Intact eggs can be contaminated with *S.* Enteritidis through infection of reproductive organs (Keller et al., 1995) or by penetration of the shell (De Reu et al., 2006). More importantly, *S.* Enteritidis can survive and multiply in eggs at concentrations that are as low as 2 CFU/mL without changing the color, smell, or consistency of egg contents (Cogan et al., 2004; Humphrey and Whitehead, 1993). Additionally, *S.* Enteritidis is generally found more frequently in the egg white of naturally contaminated eggs than in the egg yolk (Gast et al., 2002). Previous study has demonstrated that egg white is a hostile environment for bacterial survival due to its unfavourable alkalinity, nutritional limitations, and antimicrobial molecules such as lysozyme, ovotransferrin and peptides (Baron et al., 2016). Nonetheless, *S.* Enteritidis can survive and persist under these conditions, leading to epidemiological outbreaks associated with eggs. Thus, it is crucial to understand the survival mechanisms of *S.* Enteritidis in egg white.

Primary molecular biological approaches have been used to explore the survival mechanisms of *S.* Enteritidis in egg white in recent years. These methods include site-directed mutagenesis (Lu et al., 2003), transposon-mediated insertional mutagenesis (Clavijo et al., 2006), *in*

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vivo expression (Gantois et al., 2008), transposon library screening (Raspoet et al., 2014), and DNA array (Baron et al., 2017; Jakočiūnė et al., 2016). Genes identified in these studies are primarily involved in iron transport, biotin synthesis, amino acid and nucleic acid metabolism, lipopolysaccharides (LPS) biosynthesis, envelope-stress response, DNA repair, energy metabolism, motility, and pathogenicity. Furthermore, mutagenic analysis has been conducted to determine the role of some genes (*i.e. yafD*, *entF*, *tonB*, *feoAB*, *rfbH*, *rfbI*, *asnA/B*, *serA and gdhA*) in *S*. Enteritidis survival in egg white or whole egg (Gantois et al., 2009; Jakočiūnė et al., 2016; Kang et al., 2006; Lu et al., 2003). However, the above-mentioned investigations are mainly focused on elucidating the global response of *S*. Enteritidis to egg white at the transcriptional level. To our knowledge, no information is available regarding characterization of the molecular mechanisms involved in *S*. Enteritidis survival in egg white at the proteomics level.

Proteomics can efficiently provide global physiological profiles of bacteria at the protein level and offer key information about how genomic characteristics are expressed (Arunima et al., 2017). Recently, proteomic approaches have been demonstrated effective in determining the key proteins that are involved in the survival of bacteria in food matrices such as ready-to-eat meat (Mujahid et al., 2008), milk (Lippolis et al., 2009), and soy milk (Wang et al., 2013). The traditional gel-based methods such as two-dimensional gel electrophoresis, two-dimensional difference gel electrophoresis and label free method suffer from their lack of proteome coverage, sensitivity and reproducibility (Wu et al., 2006). A novel approach, isobaric tags for relative and absolute quantitation (iTRAQ), overcomes the above-mentioned shortcomings. More than one sample can be analyzed simultaneously together with biological and technical replicates (4 or 8 plex). High-resolution mass spectrometry analyses provide accurate relative ratios between protein concentrations in different samples (Wu et al., 2006). Hence, the aim of this study was to understand the survival mechanisms of *S*. Enteritidis in egg white at the proteomics level by iTRAQ technology and mutagenic analysis.

2. Materials and methods

2.1. Raw sterile liquid egg white preparation

Specific pathogen free (SPF) eggs were obtained from Meiliyaweitong Experimental Animal Technology Co., Ltd. (Beijing, China). Egg surfaces were sterilized with 70% ethanol under sterile conditions. The egg white was collected in a sterile bag after cracking the shell, homogenized for 5 min and then centrifuged at 12,000 ×*g* for 5 min at 4 °C. The supernatant with a pH value of 9.3 ± 0.1 was used in subsequent experiments.

2.2. Bacterial cultivation and sample preparation

S. Enteritidis strain SJTUF10978, originally isolated from chicken wings, was used in this study due to its high survival ability in egg white as demonstrated in our preliminary test. This strain was maintained in 50% (v/v) glycerol at −80 °C and propagated twice overnight at 37 °C on Luria-Bertani (LB) agar before use. Bacteria were freshly cultured overnight in LB broth and the culture (1 mL) was centrifuged at 13,524 ×*g* for 2 min. The supernatant was discarded and the cell pellet was suspended in 10 mL of LB broth containing various concentrations of egg white (0%, 25%, 50% and 80%), and in 10 mL of whole egg white, respectively. The initial concentration was approximately 1×10^8 CFU/mL. The samples were incubated at 37 °C and the optical density (OD_{600nm}) was measured every hour. The *S*. Enteritidis cells in LB broth (control), 50%, 80% and whole egg white samples were harvested after 6 h incubation by centrifugation (13,524 ×*g*, 5 min and 4 °C) and washed with phosphate buffer saline (PBS, pH 7.4) to move flocculent precipitation. Cell pellets were stored at −80 °C for proteomic analysis.

2.3. Total protein extraction and quantification

Total protein of *S*. Enteritidis was extracted based on a previous method with minor modifications (Wiśniewski et al., 2009). Each sample was solubilized in 500 μL SDT buffer (4% SDS, 1 mM DTT in 150 mM Tris-HCl, pH 8.0) and boiled for 5 min. The suspensions were ultrasonicated (80 w, 10 s ultrasonic at a time, every 15 s, 10 times) and centrifuged at 14,000 ×*g* for 10 min. The protein concentrations were determined using the BCA Protein Assay Reagent (Promega, Madison, WI). The extracted protein was evaluated by 12.5% SDS-PAGE gel. The supernatants were stored at −80 °C until use. Two independent growth experiments were performed to ensure reproducibility.

2.4. Protein digestion and iTRAQ labeling

Protein digestion was performed according to the filter-aided sample preparation (FASP) procedure (Wiśniewski et al., 2009). Briefly, 200 μg of protein for each sample was incorporated into 30 μL SDT buffer. The detergent, DTT and other low-molecular-weight components were removed using UA buffer (8 M Urea, 150 mM Tris-HCl pH 8.0) by repeated ultrafiltration steps (Pall Industries, 10 kDa cutoff). Iodoacetamide (0.05 M) in 100 μL UA buffer was added to the filtration unit and incubated for 30 min in darkness. The filters were washed three times with 100 μL UA buffer and then twice with 100 μL DS buffer (50 mM triethylammonium bicarbonate, pH 8.5). Finally, the protein suspensions were digested with 2 μg trypsin (Promega) in 40 μL DS buffer overnight at 37 °C and the resulting peptides were collected as a filtrate. The peptide content was estimated by UV optical density at 280 nm.

The resulting peptide mixture was labeled using the 8-plex iTRAQ reagents (113, 114, 115, 116, 117, 118, 119, 121) based on the manufacturer's instructions (Applied Biosystems, Foster City, CA). Each iTRAQ reagent was dissolved in 70 μL of ethanol and added to the respective peptide mixture. Peptides from LB, 50% egg white, 80% egg white, and whole egg white were labeled with iTRAQ reagents containing the reporters 113, 115, 117, and 119, respectively. Additional independent biological replicates were labeled with other reagents containing the reporters 114, 116, 118, and 121, respectively. After labeling, samples were multiplexed and vacuum dried.

2.5. Peptide fractionation with strong cation exchange (SCX) chromatography

The iTRAQ labeled peptides were fractionated by SCX chromatography using the AKTA Purifier system (GE Healthcare, Sweden). The dried peptide mixture was reconstituted and acidified with 2 mL buffer A (10 mM KH_2PO_4 in 25% acetonitrile, pH 3.0) and chromatographed using a strong cation exchange column (PolySulfoethyl, 4.6 × 100 mm, 5 μm, PolyLC Columbia, MD). The peptides were eluted at a flow rate of 1 mL/min using a gradient of 0%–10% buffer B (500 mM KCl, 10 mM KH_2PO_4 in 25% acetonitrile, pH 3.0) for 32 min, 10–20% buffer B for 10 min, 20%–45% buffer B for 5 min, and 45%–100% buffer B for 13 min. Peptide elution was monitored by absorbance at 214 nm and fractions were collected every 1 min. A total of 36 fractions were combined into 15 pools and desalted on C_{18} cartridges (Empore SPE Sigma, St. Louis, MO). The fractions were concentrated by vacuum centrifugation and reconstituted in 40 μL of 0.1% (v/v) trifluoroacetic acid. All samples were stored at −80 °C until LC-MS/MS analysis.

2.6. LC-electrospray ionization (ESI)-MS/MS analysis

Mass spectroscopy experiments were performed using a Q Exactive mass spectrometer coupled to an Easy nLC liquid chromatography system (Proxeon Biosystems, Thermo Fisher, Fairlawn, NJ). Sample of each fraction (10 μL) was loaded onto a C_{18}-reversed phase column (15 × 75) packed in-house with RP-C_{18} 5 μm resin in buffer A (0.1%

formic acid) and separated using a linear gradient of buffer B (80% acetonitrile and 0.1% formic acid) at a flow rate of 250 nL/min over 140 min. MS data was acquired using a data-dependent top 10 method dynamically choosing the most abundant precursor ions from the survey scan (300–1800 *m/z*) for higher energy collisional dissociation (HCD) fragmentation. Determination of the target value was predicted using the Automatic Gain Control software provided with the instrument using dynamic exclusion duration of 60 s. Survey scans were acquired at a resolution of 70,000 at m/z 200 and resolution for HCD spectra was set to 17,500 at m/z 200. Normalized collision energy was 30 eV and the under-fill ratio that specifies the minimum percentage of the target value likely to be reached at maximum fill time, was defined as 0.1%. The instrument was run with peptide recognition mode enabled.

2.7. Sequence database searching and data analysis

MS/MS spectra were searched using MASCOT engine (Matrix Science, London, UK; version 2.2) embedded into Proteome Discoverer 1.4 (Thermo Electron, San Jose, CA) against the uniprot *Salmonella* Enteritidis (37,314 sequences, downloaded on June 22th, 2016) and the decoy database. For protein identification, the following options were used: peptide mass tolerance = 20 ppm, MS/MS tolerance = 0.1 Da, enzyme = Trypsin, missed cleavage = 2, fixed modification: carbamidomethyl (C), iTRAQ 8plex (K), iTRAQ 8plex (N-term), Variable modification: oxidation (M), FDR ≤ 0.01. The threshold for differentially expressed proteins was set as fold change > 1.5 or < 0.67 and $P < 0.05$.

2.8. Bioinformatics analysis

The Gene Ontology (GO) program Blast2GO (https://www.blast2go.com/) was used to annotate differentially expressed proteins to create histograms of GO annotation groups including cell component, biological process and molecular function (Götz et al., 2008). For pathway analysis, the differentially expressed proteins were mapped to the terms in the KEGG database (Kyoto Encyclopedia of Genes and Genomes) using the KAAS program (http://www.genome.jp/kaas-bin/kaas_main) (Kanehisa et al., 2012). Protein-protein interaction networks were analyzed using STRING (http://string-db.org/) and minimum required interaction score was set at 0.400.

2.9. Gene expression analysis

Thirty differentially expressed proteins commonly affected by all treatments of egg white were used to analyze the corresponding transcriptional profiles *via* RT-qPCR. The oligonucleotide primers used were listed in Table S1. Total RNA was extracted from the *S*. Enteritidis cells harvested from control, 50%, 80% and whole egg white samples at 6 h using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. The concentration of RNA was determined by a Nanodrop 2000c spectrophotometer (Thermo Scientific, South Logan, UT). The RNA integrity was evaluated by 1% agarose gel electrophoresis. DNase treatment and synthesis of cDNA from RNA were carried out using the PrimeScript RT reagent kit with gDNA Eraser (Takara, Dalian, China). First strand cDNA was used as template for real time PCR using SYBR Premix Ex Taq II reagents (Takara, Dalian, China) using 2 μL of cDNA and 0.5 μL of each primer (10 μM) in a final volume of 20 μL. Thermal cycling conditions were as follows: 95 °C/5 min, 40 cycles of 95 °C/15 s, 58 °C/15 s, 68 °C/30 s. Melting curve analysis of amplified products was performed to confirm that only the target product was amplified using software supplied with the instrument. 16S rRNA was chosen as a reference gene to normalize the expression of the tested genes (He et al., 2018). Three biological replicates were used and relative gene expression was calculated using the $2^{-\triangle\triangle CT}$ threshold cycle (C_T) method (Livak and Schmittgen, 2001). The gene was regarded as up-regulated when the relative expression level was over two-fold (He et al., 2018).

2.10. Construction of the ybgC mutant strain of S. Enteritidis and complementation

Mutant construction was conducted according to a published method (Zhou et al., 2014) using oligonucleotide primers listed in Table S2. Strains and plasmids used in this study were listed in Table S3. The constructed plasmid pRE△*ybgC*-C was transferred into the △*ybgC* mutant strain by electroporation. The complemented strain was analyzed by PCR and DNA sequencing to confirm gene complementation.

2.11. Survival characterization of S. Enteritidis strains in egg white

Survival ability of *S*. Enteritidis in egg white was performed based on a previous method with minor modifications (Clavijo et al., 2006). Briefly, egg white was added to 96-well plates and mixed with overnight cultures of bacterial strains to give a final concentration of 10^3 and 10^6 CFU/mL. The mixtures were incubated at 37 °C for 6 h and 24 h. After incubation, viable bacteria were counted by plating the cells on LB agar. Three independent experiments were completed. There were three replicates in each experiment.

2.12. Scanning electron microscopy

Cell morphology of *S*. Enteritidis strains in LB broth and egg white was observed by scanning electron microscopy (SEM) as previously described (He et al., 2016).

2.13. Expression analysis of the tol-pal system-related genes

The expression of the *tol-pal* system-related genes (*tolQ*, *tolR*, *tolA*, *tolB*, *pal*, *ybgF*) was analyzed by RT-qPCR. Total RNA was extracted from the wild type and △*ybgC* mutant strains in whole egg white at 6 h following the manufacturer's protocol. Experimental procedures were carried out according to the aforementioned method (from gene expression analysis). The oligonucleotide primers used were listed in Table S4.

2.14. Statistical analysis

All statistical analyses were performed using SAS Software. The data were analyzed by one-way analysis of variance (ANOVA). Comparison of means was performed based on Duncan's multiple range tests. Statistical significance was assessed at the level of $P < 0.05$.

3. Results and discussion

3.1. Proteomic profile of S. Enteritidis in response to egg white

The proteomic profile of pathogenic bacteria in food matrix is generally compared with that in laboratory media to elucidate the survival mechanisms (Lippolis et al., 2009; Mujahid et al., 2008). Hence, we compared the proteomic profiles of *S*. Enteritidis in different concentrations of egg white with the profile in LB broth in this study. The growth of *S*. Enteritidis in the presence of egg white was monitored by measuring OD_{600nm} under five conditions (LB broth, LB broth containing 25%, 50% and 80% egg white, and whole egg white). It was found that *S*. Enteritidis growth was inhibited with the increase of egg white concentration (Fig. 1A). The log-phase bacteria are generally utilized to extract proteins for proteomic analysis (Lippolis et al., 2009; Wang et al., 2013). In this study, a log-phase culture of *S*. Enteritidis was obtained by incubating in egg white for 6 h and the cell number was determined by plate counting. It was found that exposure to all concentrations of egg white significantly decreased the population of *S*.

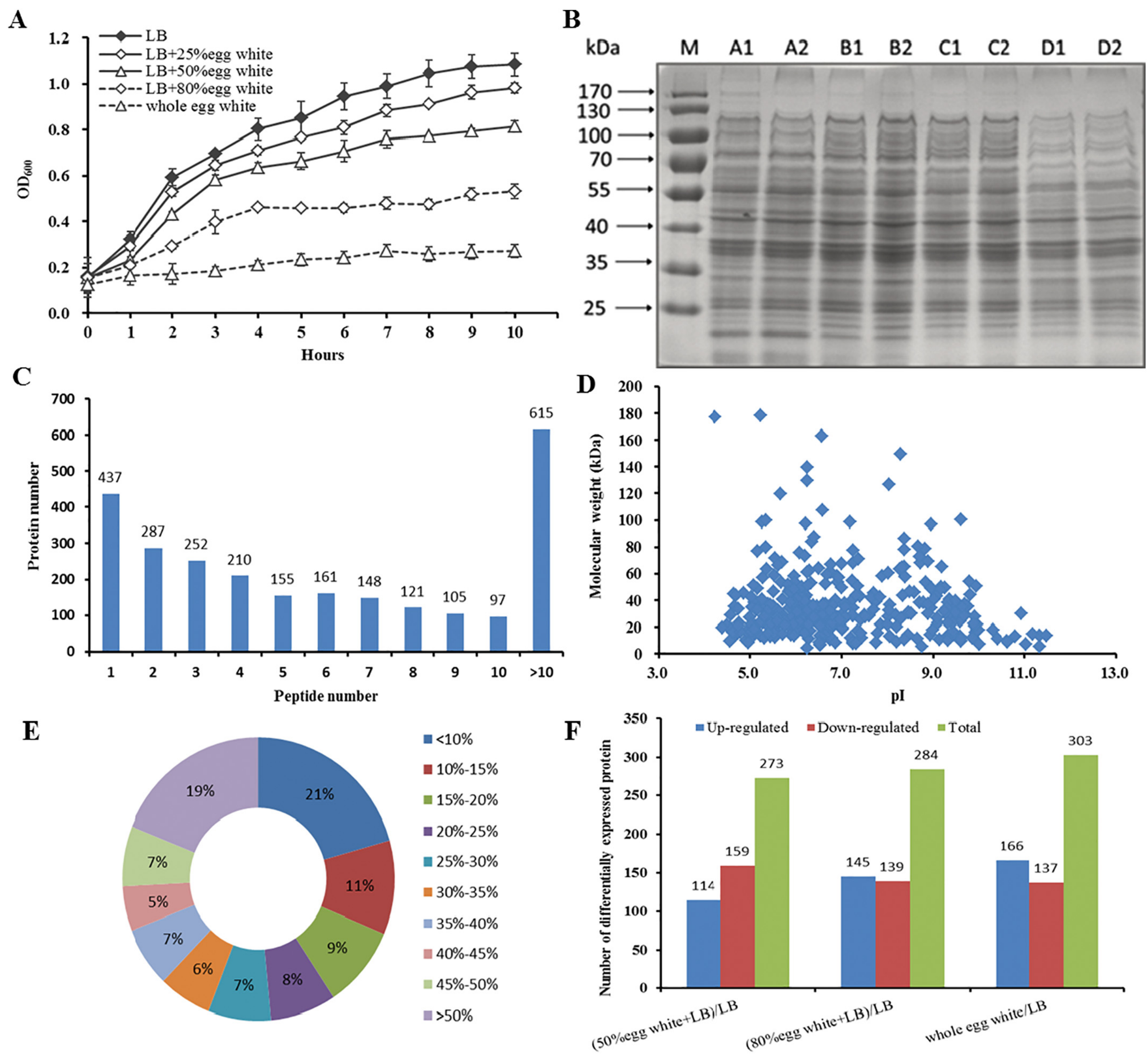

Fig. 1. Proteomic analysis of *S.* Enteritidis in response to egg white. (A) Growth curves of *S.* Enteritidis in LB broth containing 0%, 25%, 50% and 80% egg white and in whole egg white. Error bars represent the standard deviation of three replicates. (B) SDS-PAGE analysis of proteins collected from *S.* Enteritidis in 0%, 50%, 80% and whole egg white. Lanes A1 and A2, LB only; Lanes B1 and B2, 50% egg white in LB; Lanes C1 and C2, 80% egg white in LB; Lanes D1 and D2, whole egg white. (C) Number of peptides associated with identified proteins. (D) Molecular weights and isoelectric points calculated from protein sequences. (E) Sequence coverage for identified proteins. (F) Overview of differentially expressed proteins of *S.* Enteritidis in 50%, 80% and whole egg white compared with LB broth.

Enteritidis ($P < 0.05$), except for 25% egg white treatment (data not shown). Therefore, *S.* Enteritidis cells were harvested for proteomic analysis after incubation for 6 h in LB broth containing 0%, 50%, 80% egg white, and in whole egg white.

No apparent difference of the protein profile of *S.* Enteritidis in 50%, 80%, and whole egg white was observed compared with that in LB broth on SDS-PAGE, indicating that a majority of egg white proteins had been removed (Fig. 1B). A total of 313,055 spectra, 21,650 peptides and 2588 proteins were detected and identified by iTRAQ. Over 83% of the proteins were represented by at least two peptides (Fig. 1C). Molecular weights ranged from 4 to 180 kDa, and isoelectric points ranged from 4.0 to 12.0 (Fig. 1D). Approximately 79% of identified proteins had > 10% peptide sequence coverage (Fig. 1E). A total of 303 (166 up-regulated and 137 down-regulated), 284 (145 up-regulated and 139 down-regulated), and 273 (114 up-regulated and 159 down-regulated) differentially expressed proteins were acquired from *S.* Enteritidis grown in whole, 80%, and 50% egg white compared with that in LB broth, respectively (Fig. 1F). A total of 93 proteins, including 25 up-regulated and 68 down-regulated, were commonly affected by all treatments of egg white (Supplementary Data S1). Details for all differentially expressed proteins were presented in Supplementary Data S2.

3.2. Validation of iTRAQ results

Transcriptional profile of 30 differentially expressed proteins was validated by RT-qPCR. Interestingly, alterations in the mRNA levels of these proteins were in accordance with those in the corresponding

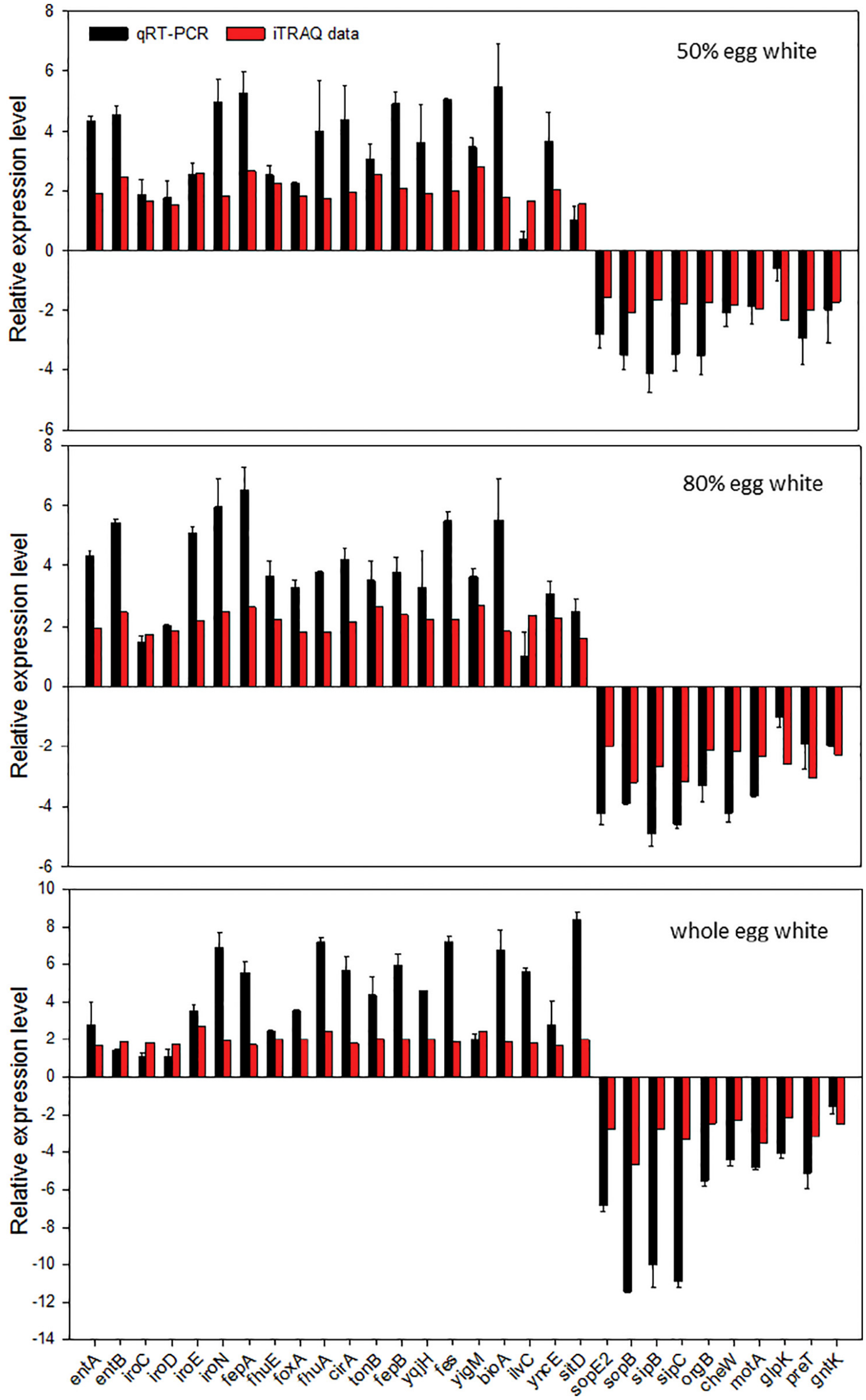

Fig. 2. Comparison of protein and mRNA levels of differentially expressed proteins revealed by iTRAQ and RT-qPCR in the presence of 50%, 80%, and whole egg white. Protein and mRNA levels were presented as fold change and Log_2ratio, respectively. Error bars indicate the standard deviation of three technical replicates.

protein levels, except for IlvC and GlpK in 50% egg white group (Fig. 2). Despite the differences in the relative expression level obtained from RT-qPCR and iTRAQ results, the overall trends in gene and protein expression levels were generally identical. These data provided an evidence for the reliability of our results on the relative quantitative protein expression.

3.3. Functional characterization of differentially expressed proteins

To obtain a better understanding of the survival mechanisms of *S.* Enteritidis in response to egg white, the differentially expressed proteins were subjected to bioinformatics analysis. GO level 2 analysis was performed to identify the biological process, cellular component, and

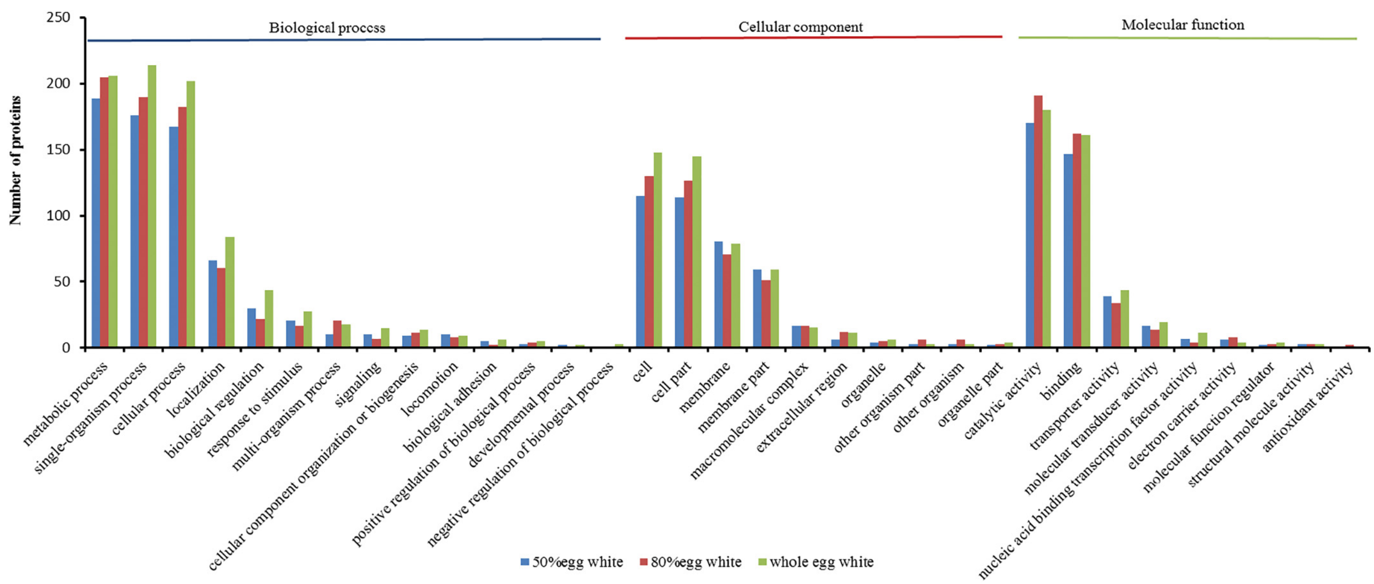

Fig. 3. Distribution of differentially expressed proteins based on their predicted biological process, cellular component and molecular function in the presence of 50%, 80%, and whole egg white.

molecular function (Fig. 3). Biological process analysis indicated that these proteins were primarily involved in metabolic process, single-organism process, cellular process and localization. Cellular components analysis indicated that these proteins were mainly located in cell and membrane. In addition, molecular function analysis showed that these proteins were mainly related to catalytic activity, binding and transporter activity (Fig. 3).

KEGG pathway analysis showed that differentially expressed proteins were primarily involved in iron acquisition, cofactor and amino acid biosynthesis, energy metabolism, transporter, regulation, virulence, motility and chemotaxis as well as stress responses. Approximately 10% of identified proteins were grouped to uncharacterized proteins. The expression patterns of these proteins were shown in Fig. 4. The protein-protein interaction networks of these proteins displayed a close interaction among pathways (Fig. 5), indicating that a coordinated regulation of different pathways was involved in the survival of *S.* Enteritidis in egg white. Furthermore, a proposed model based on the proteomics result was further formulated for a better understanding of the survival strategies employed by *S.* Enteritidis to survive in egg white (Fig. 6).

3.3.1. Iron acquisition-related proteins

Iron-deficient environment is unfavourable for bacterial growth and survival and iron chelation by ovotransferrin is a key antimicrobial activity of egg white (Kang et al., 2006). In this study, marked changes in proteins involved in iron acquisition system in *S.* Enteritidis were found in the presence of egg white (Figs. 4; 6). Proteins (EntABCEFH and IroBCDEN) involved in biosynthesis and export of enterobactin and salmochelin were significantly up-regulated. Additionally, numerous outer membrane receptors involved in ferric-enterobactin, ferric-hydroxamate and ferric-ferrioxamine uptake were up-regulated, including FepA, CirA, FhuA, FhuE and FoxA. The TonB-ExbB-ExbD complex for energy-dependent transport of ferric siderophores and the periplasmic-binding proteins (FepB, FepC, YncE and YqjH) involved in Fe^{3+} siderophore transport were also induced (Figs. 4; 6). *Salmonella* has siderophore-mediated iron uptake systems and can synthesize and secrete siderophores enterobactin and salmochelin (Neilands, 1995). Enterobactin can be glycosylated to produce salmochelin *via* the *iro* operon including glycosyltransferase (IroB), inner membrane efflux pump (IroC), esterases (IroD and IroE) for degradation of ferric-salmochelins and a ferric-salmochelin receptor (IroN) (Bäumler et al., 1998). It has been shown that the uptake of ferric-siderophore complexes in Gram-negative bacteria includes specific outer membrane receptors, energy-transducing systems and periplasmic binding protein dependent ATP-binding cassette transporters (Andrews et al., 2003).

The expression pattern of iron acquisition-related proteins revealed in this study is consistent with that of gene expression in the presence of 10% egg white found by Baron et al. (2017). Ferric and ferrous iron-acquisition genes such as *entF*, *tonB* and *feoAB* have been shown to be important for *S.* Enteritidis survival in egg white (Kang et al., 2006). Thus, it is indicative that the iron acquisition system of *S.* Enteritidis is essential for its survival in egg white. However, in our data, no significant difference of survival ability between mutants (△*iroD* or △*fes*) and wild type strains was shown in egg white ($P > 0.05$) (Fig. S1). In *Escherichia coli* (*E. coli*), the growth of △*fes*△*iroD* and △*fes*△*iroD*△*iroE* mutants has been shown to be severely reduced in the presence of ovotransferrin (Caza et al., 2015). Hence, we suggest that a single gene mutant of esterase (*iroD* or *fes*) of *S.* Enteritidis is not necessary for its survival in egg white. The bacterium can use other esterases to obtain iron for iron homeostasis in cells, when one esterase (*iroD* or *fes*) is absent.

3.3.2. Cofactor and vitamin biosynthesis-related proteins

Proteins involved in cofactor and vitamin biosynthesis were up-regulated in *S.* Enteritidis exposed to egg white (Figs. 4; 6). The expression of biotin biosynthesis proteins (BioA, BioB and BioD) was strongly induced (1.60- to 4.00-fold). Additionally, the expressions of thiamine kinase, quinolinate synthase and L-aspartate oxidase were up-regulated in whole egg white. Egg white contains several vitamin-chelating proteins such as avidin, thiamine and riboflavin-binding proteins (Baron et al., 2016). These proteins are regarded as a part of the defence system of egg white against bacteria through chelating vitamins that are essential for life. Avidin prevents the growth of bacteria due to its high affinity for biotin, and biotin synthesis-related gene *bioB* has been shown to be implicated in *S.* Enteritidis survival in egg white (Raspoet et al., 2014). Our data indicate that the up-regulation of cofactor and vitamin-related proteins correlates with the poor vitamin availability imposed by egg white.

3.3.3. Amino acid biosynthesis-related proteins

A considerable proportion of up-regulated proteins was associated with amino acid biosynthesis with whole egg white exposure (1.63- to

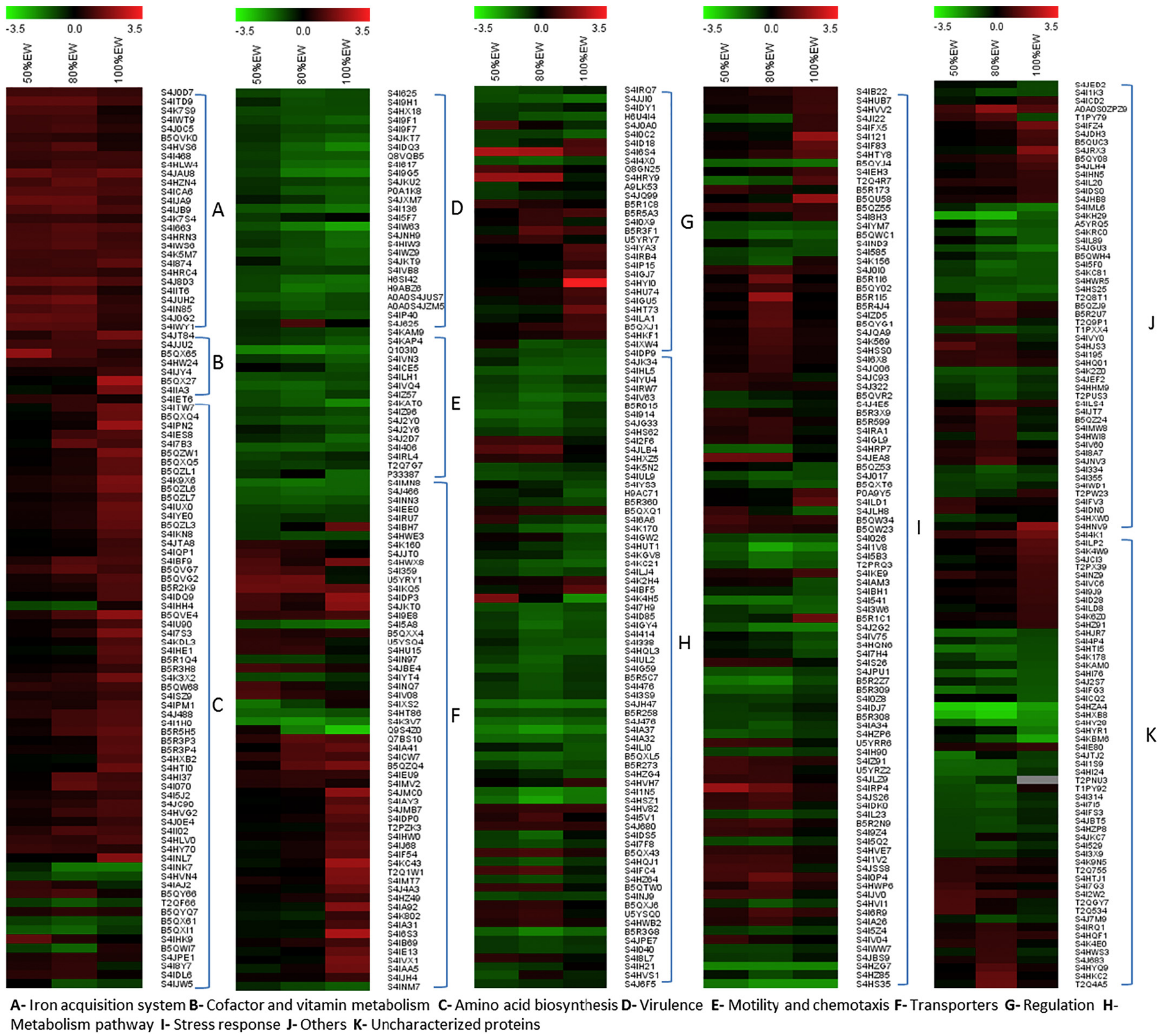

Fig. 4. Heat map of differentially expressed proteins identified from egg white exposure (FDR ≤ 0.01). Each column represents the different concentrations of egg white (EW), 50%, 80%, and 100% (whole) egg white, as indicated. Each row represents individual protein. The scale of this heat map is given as Log_2fold change, ranging from −3.5 (green) to +3.5 (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4.21-fold; Figs. 4; 6). These amino acids included alanine, aspartate, glutamate, glycine, serine, threonine, cysteine, methionine, lysine, arginine, histidine as well as branched chain and aromatic amino acids. This result is consistent with the fact that LB contains a rich supply of free amino acids (yeast extract) that are not available in whole egg white. In contrast, the vast majority of genes involved in amino acid biosynthesis have been shown to be repressed by exposure of *S*. Enteritidis to 10% egg white at 45 °C (Baron et al., 2017). The difference may be that 45 °C is a bactericidal temperature causing a reduction in amino acid biosynthesis. Are amino acid biosynthesis-related proteins essential for *S*. Enteritidis survival in egg white? The lysine and aromatic metabolism-related mutants have been shown to be more susceptible to egg white than the wild type strain (Clavijo et al., 2006). Additionally, it has also been shown that genes involved in asparagine (*asn*), arginine (*pyr*, *car*), serine (*ser*), leucine (*leu*) and isoleucine (*ilv*) are up-regulated in *S*. Enteritidis upon exposure to whole egg, but these highly expressed genes (*asnA/B*, *serA and gdhA*) are not essential for its growth in whole egg (Jakočiūnė et al., 2016). It is difficult to compare these results because the experimental conditions (*i.e*. egg white, whole egg containing egg yolk) vary in different reports.

3.3.4. Energy and carbohydrate metabolism-related proteins

The expression of a certain percentage of proteins associated with energy metabolism in *S*. Enteritidis was altered in the presence of egg white (Figs. 4; 6). Fumarate reductase (FrdA, FrdB, FrdC and FrdD) and fumarate hydratases (FumA and FumB) were down-regulated. These enzymes have been shown to catalyze the reaction that converts fumarate into malic acid in the seventh step of TCA cycle (Takeuchi et al., 2015). Many proteins involved in electron transport chain, including hydrogenases (HypO, HybA, HybC, STM1534 and STM1531) and cytochromes (STM1792 and STM1537), were also suppressed in this study. Similarly, the energy metabolism-related genes have been shown to be down-regulated at the transcriptional level (Baron et al., 2017). An energy conservation strategy would reduce ATP and proton motive

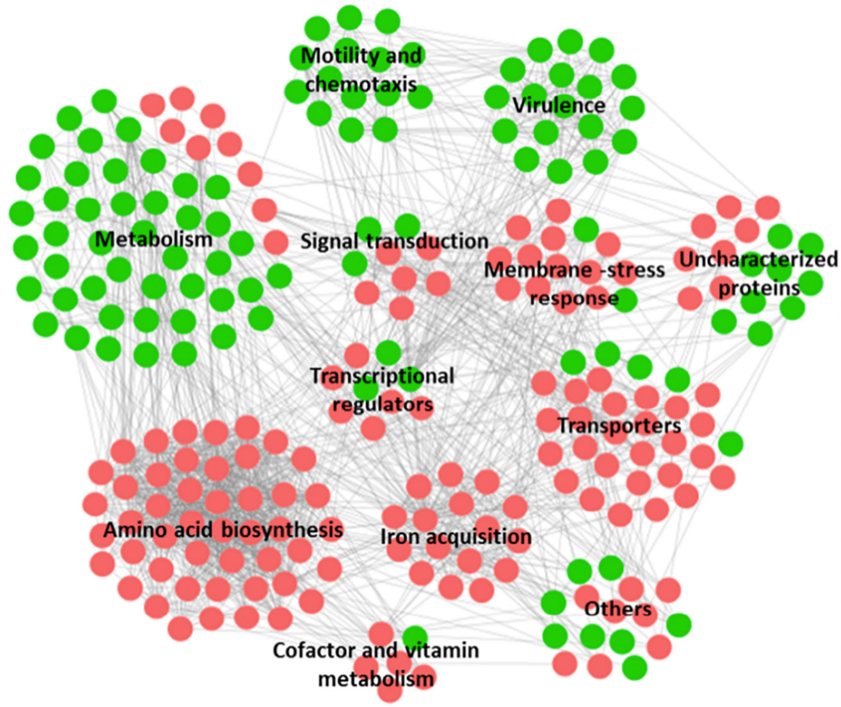

Fig. 5. Interaction networks of differentially expressed proteins with whole egg white exposure using cytoscape 3.5.1 software. Red cycles represent the up-regulated proteins and green cycles represent the down-regulated proteins. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

force, leading to a survival advantage (Minamino et al., 2014). Hence, we suggest that cells reduce their energy demand by decreasing the activity of central metabolism (glycolysis, tricarboxylic acid cycle, pentose phosphate pathway and glycogenesis) under nutrient deprivation stress of egg white.

3.3.5. Transporter and regulation-related proteins

The expression of transporter related-proteins (SitA, SitB, SitC and SitD) was up-regulated in the presence of egg white (Figs. 4; 6). The *sit* operon has been shown to encode an iron and manganese uptake system that is activated in alkaline as well as low iron conditions (Bippes et al., 2013). In our data, some transporters such as the ammonium transporter (AmtB), amino acid permease (STM2359) and amino acid transporters (HisMPJ, GlnHPQ, GltI, etc.) were induced in whole egg white. These amino acid transporters were involved in histidine, glutamine, leucine, lysine, arginine, serine, and glutamate physiology, which correlated well with the upregulation of amino acid biosynthesis-related proteins. In addition, the phosphate specific transport system-related proteins (PhoU, PstS and PstB) and the PhoR/B two-component system involved in the regulation of phosphate transport were also up-regulated in whole egg white. Phosphate is crucial for the synthesis of membrane lipids and nucleic acids as well as signal transduction (Santosbeneit, 2015). Upon phosphate limitation, the two-component system (PhoR/PhoB) has been shown to regulate expression of > 30 genes belonging to the *pho* regulon (Hsieh and Wanner, 2010). Moreover, in our data, the peptide permease (SapB) was significantly induced in response to whole egg white. The SapABCDF system mediates resistance to antimicrobial peptides by internal transport for either degradation or initiation of a regulatory cascade that activated resistance determinants (Parralopez et al., 1993). It is likely that the up-regulated protein (SapB) is induced by antimicrobial peptides of egg white. Hence, the up-regulation of all above-mentioned transporter-related proteins correlate well with the adverse environment (*i.e.* alkaline, low iron and phosphate, amino acid limitations and antimicrobial peptides) imposed by egg white.

Marked changes in proteins involved in two-component systems and transcriptional regulatory in *S.* Enteritidis were found in the presence of egg white (Fig. 6), including up-regulated proteins (GlnL/GlnG, PhoR/PhoB, CpxR, TorS, MetR, SgrR, IscR, ArgR, LrP) and down-regulated proteins (RpoS and HilA). LrP suppresses several genes including *phoP* and *rpoS* that regulates virulence-related genes (Fang et al., 1992). The protein HilA is an activator of *Salmonella* Pathogenicity Island 1 (SPI1) and the regulator of *hilA* is Fur, which is required for virulence in *S.* Typhimurium and activation of *hilA* and the *hilA*-dependent genes *invF* and *sipC* (Troxell et al., 2011).

3.3.6. Virulence, motility and chemotaxis-related proteins

In this study, virulence-related proteins were down-regulated in the presence of egg white (1.67- to 4.76-fold; Fig. 6), which were consistent with the down-regulation of HilA and RpoS. Some of them were members of SPI1, including InvB, InvC, InvJ, OrgB, PrgH, PrgI, SipA, SipB, SipC, SipD, SpaO, SopB, SopD and SopE as well as the plasmid proteins (SpvR, SpvA, SpvB, SpvC and SpvD). The *spv* locus includes a LysR-type regulatory protein (SpvR) that regulates expression of the *spvRABCD* operon (Lahiri et al., 2009). SPI1 expression has been shown to be modulated by several environmental factors such as alkalinity and low iron (Bajaj et al., 1996). Similarly, proteins involved in motility and chemotaxis were also down-regulated in the presence of egg white (1.61- to 3.85-fold; Fig. 6), including FliB, FliC, FliD, FliH, FliN, FliL, FlgM, MotA, MotB, CheW, CheZ, and Aer. Previous works have indicated that deletion of motility-related genes (*fliC*, *motAB*, *flgG*) decreases the survival ability of *S.* Enteritidis in egg albumen (Cogan et al., 2004; Gantois et al., 2008). It has also been shown that virulence and motility-related genes are down-regulated by exposure of *S.* Enteritidis to 10% egg white (Baron et al., 2017). Moreover, the expression of flagella, motility, and chemotaxis components (all of which contribute to virulence) is highly regulated by multiple environmental stresses such as extreme pH, iron restriction and cell membrane stress (Maurer et al., 2005; Ryan et al., 2015).

3.3.7. Stress response-related proteins

A definite proportion of stress response-related proteins were regulated in the presence of egg white. These proteins were primarily related to DNA repair, redox process, and cell membrane (Fig. 6). Two enzymes involved in DNA repair, single-stranded DNA specific exonuclease (RecJ) and crossover junction endodeoxyribonuclease (RusA),

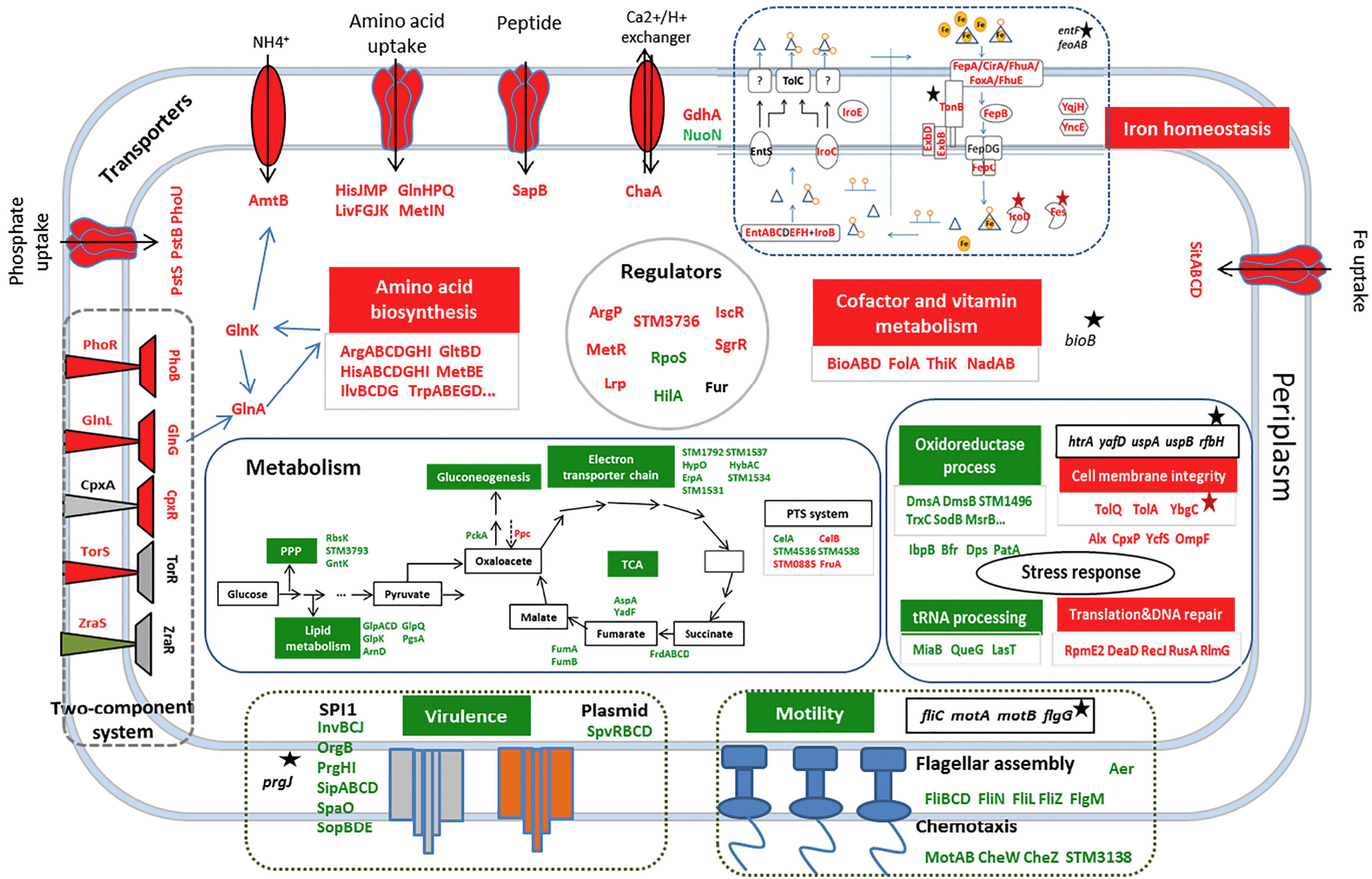

Fig. 6. Proposed model for survival of *S.* Enteritidis in egg white. Red (up-regulated), green (down-regulated) and black (non-regulated) were presented. Black star (★) represents the mutant genes validated according to the references. Red star (☆) represents the mutant genes validated in this study. Boxes in red and green are the up-regulated and down-regulated proteins associated with functional classification, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

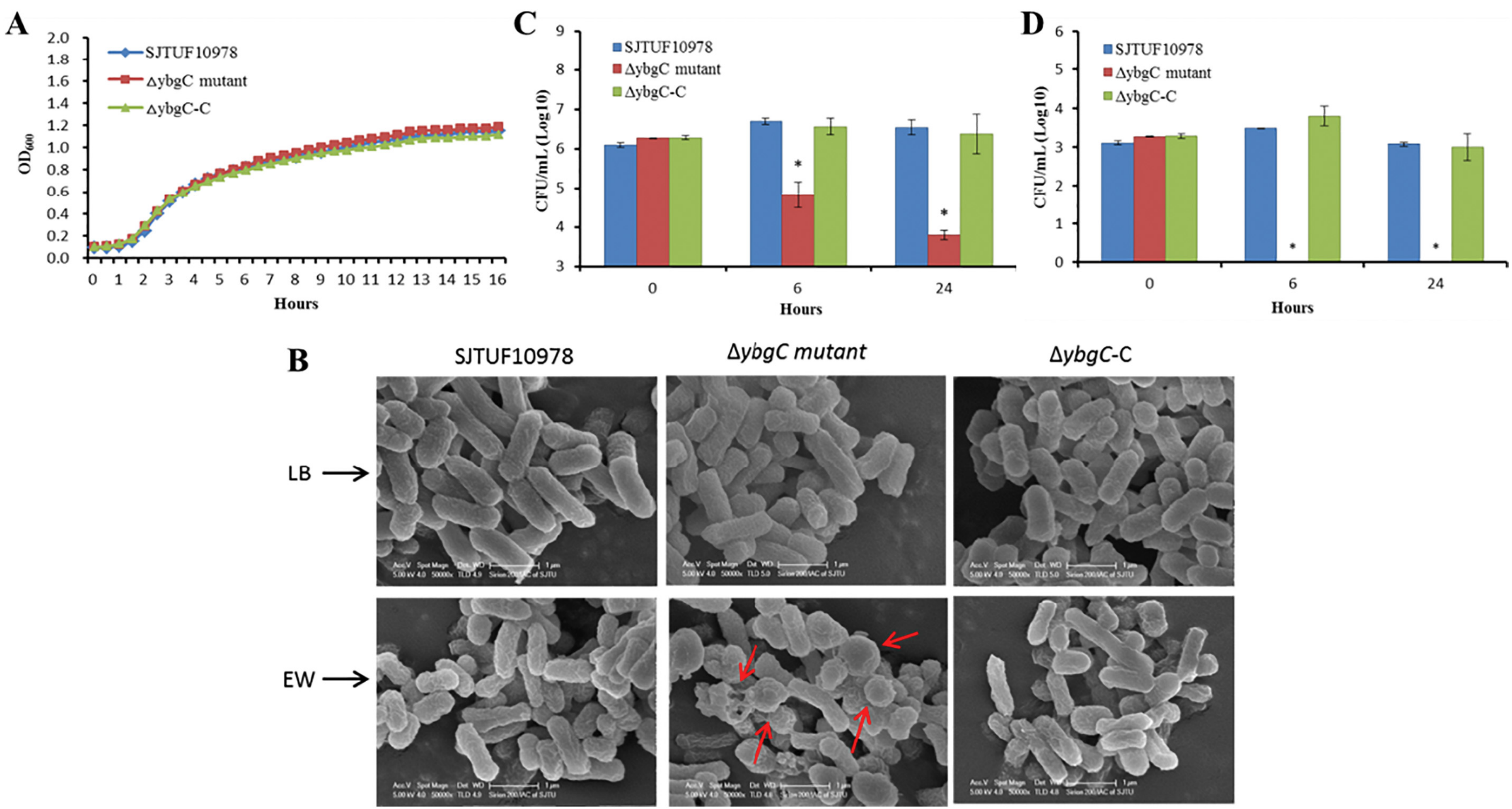

Fig. 7. Survival characteristics of *S.* Enteritidis wild type, △*ybgC* mutant and △*ybgC*-C complementary strains in egg white. (A) Growth curves of *S.* Enteritidis wild type, △*ybgC* mutant and △*ybgC*-C complementary strains in LB broth. (B) SEM micrographs of wild type, △*ybgC* mutant and △*ybgC*-C complementary strains in LB broth and whole egg white. Red arrows highlight clear examples of morphology changes. Magnification = 50,000×, bar marker = 1 μm. Survival assays of wild type, △*ybgC* mutant and △*ybgC*-C complementary strains in whole egg white with an initial concentration of 10^6 CFU/mL (C) and 10^3 CFU/mL (D). At least three experiments were performed and the results of representative experiments were shown. Error bars indicate the standard deviation of three technical replicates. All asterisks indicate statistical differences according to Duncan's multiple range test at $P < 0.05$ level. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

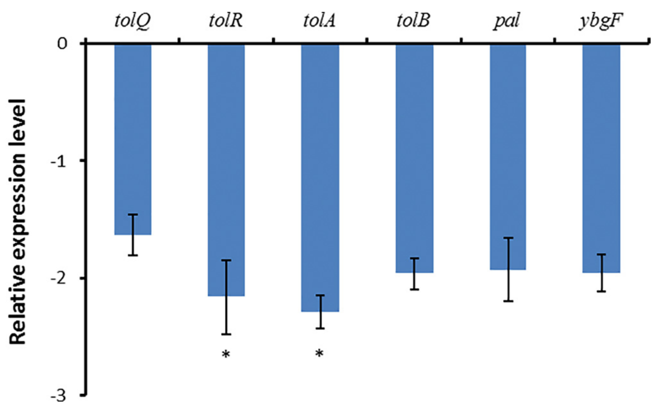

Fig. 8. Relative expression levels of *tol-pal* system-related genes of wild type and △*ybgC* mutant strains in whole egg white. Error bars indicate the standard deviation of three technical replicates. All asterisks indicate statistical differences according to Duncan's multiple range test at $P < 0.05$ level.

were significantly induced by whole egg white ($P < 0.05$). Previous study has indicated that *yafD* is homologous to members of an exonuclease-endonuclease-phosphatase family and it provides a survival advantage to *S.* Enteritidis in eggs by repairing DNA damage caused by egg white (Lu et al., 2003). These results suggest that there is a repair system in *S.* Enteritidis to respond to egg white. Additionally, in this study, some proteins involved in oxidoreductase process were down-regulated in egg white, including thioredoxin (TrxC), reductases (DmsA, DmsB, DmsC and STM1497), FAD dependent oxidoreductase (STM2175), bacterioferritin (Bfr), DNA protection during starvation protein (Dps), and superoxide dismutase (SodB). Bfr is required for capture of free Fe^{2+} under iron-rich conditions and its subsequent conversion to Fe^{3+} for storage in its cavity (Yao et al., 2012). Similarly, Dps sequesters iron and limits fenton-catalyzed oxyradical formation and its expression has been shown to be regulated by the stationary phase sigma factor RpoS as well as OxyR and IHF (Altuvia et al., 1994). These effects correlate well with the iron-restriction imposed by egg white. In addition, in this study, cell membrane-related proteins were also up-regulated by whole egg white, such as L,D transpeptidase (YcfS), inner membrane multidrug efflux protein (AcrD), inner membrane protein (Alx), outer membrane protein (OmpF), maltoporin (LamB) and *tol-pal* system-related proteins (YbgC, TolQ, TolA). These genes have been shown to be up-regulated by 10% egg white at the transcriptional level (Baron et al., 2017). However, LPS biosynthesis-related proteins were not identified in this study. Previous studies have found that deletion of LPS biosynthesis-related genes increases susceptibility of *S.* Enteritidis to egg white (Gantois et al., 2009; Raspoet et al., 2014). This result may be related to difference in experimental conditions such as temperature, inoculum size and incubation time.

3.4. YbgC was crucial for S. Enteritidis survival in egg white

The *tol-pal* system, maintaining the outer-membrane integrity of Gram-negative bacteria, is first described in *E. coli* (Sun and Webster, 1987), and more recently in several other species (Llamas et al., 2000; Prouty et al., 2002). The *tol-pal* system consists of six proteins, including inner membrane protein TolA, TolQ, and TolR, periplasmic proteins TolB and YbgF, and outer membrane protein Pal (Bouveret et al., 1999). However, the *tol-pal* gene cluster is commonly comprised of seven open reading frames, *ybgC-tolQ-tolR-tolA-tolB-pal-ybgF*. Acyl-CoA thioesterase activity for YbgC in *E. coli* and *Helicobacter pylori* has been demonstrated, and it has been proposed that YbgC may be involved in the biosynthesis of species-specific phospholipids (Angelini et al., 2008; Gully and Bouveret, 2006). However, the role of this protein in *S.* Enteritidis survival in egg white is not clear. Hence, in this study, gene (*ybgC*) was deleted to confirm its role for *S.* Enteritidis survival in egg white.

The wild type, △*ybgC* mutant and △*ybgC*-C complementary strains exhibited similar growth patterns in LB broth at 37 °C (Fig. 7A). No significant morphological change was found among these strains (Fig. 7B). These results indicate that *ybgC* is not necessary for *S.* Enteritidis growth in LB broth. Interestingly, the survival ability of △*ybgC* mutant strain was significantly lower than the wild type and △*ybgC*-C complementary strains ($P < 0.05$) in whole egg white at 6 h and 24 h (Fig. 7C and D). A 3-log population reduction was observed in △*ybgC* mutant strain after incubation in whole egg white for 24 h with an initial concentration of 10^6 or 10^3 CFU/mL (Fig. 7C and D). No significant difference between the wild type and △*ybgC*-C complementary strains ($P > 0.05$) was observed. The cellular morphology of △*ybgC* mutant strain exposed to whole egg white was altered from a rod to a spherical shape along with cell lysis, compared with that of wild type strain (Fig. 7B). No significant morphological changes between the wild type and △*ybgC*-C complementary strains were observed in whole egg white (Fig. 7B). These results indicate the crucial role of *ybgC* for *S.* Enteritidis survival under egg white stress.

3.5. Deletion of ybgC decreased the expression of tolR and tolA

To date, there is no evidence to support a role of YbgC in the *tol-pal* system (Sturgis, 2001). To explore the role of *ybgC*, the expression of the *tol-pal* system-related genes (*tolQ*, *tolR*, *tolA*, *tolB*, *pal*, *ybgF*) was analyzed in this study. Results showed that the expression of *tolR* and *tolA* in △*ybgC* mutant strain was significantly down-regulated in whole egg white compared with that in wild type strain, but no difference for *tolQ*, *tolB*, *pal* and *ybgF* was observed (Fig. 8). Therefore, we speculate that deletion of *ybgC* may affect the *tol-pal* system that maintaining the cell membrane integrity, thus leading to decreased survival of *S.* Enteritidis in egg white.

4. Conclusion

Proteomic characterization indicates multiple strategies employed by *S.* Enteritidis to survive in egg white, including the induction of iron acquisition and cofactor biosynthesis, enhanced biosynthesis and transporter of amino acids, a specific energy conservation survival mode, and promoted tolerance against DNA and cell membrane damage. Furthermore, YbgC (acyl-CoA thioesterase) is an essential enzyme contributing to the survival of *S.* Enteritidis in egg white. These results provide novel insights into the underlying survival mechanisms of *S.* Enteritidis in egg white at the proteomics level.

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Conflict of interest

All authors declare that they have no conflict of interest.

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