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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%

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