Journal of Proteomics 157 (2017) 52–58

Contents lists available at ScienceDirect

Journal of Proteomics

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

Salmonella proteomics under oxidative stress reveals coordinated regulation of antioxidant defense with iron metabolism and bacterial virulence

Jiaqi Fu [a,1], Linlu Qi [a,1], Mo Hu [a], Yanhua Liu [a], Kaiwen Yu [a], Qian Liu [b], Xiaoyun Liu [a,*]

[a] Institute of Analytical Chemistry and Synthetic and Functional Biomolecules Center, College of Chemistry and Molecular Engineering, Peking University, 100871 Beijing, China
[b] State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, 100085 Beijing, China

ARTICLE INFO

Article history:
Received 9 October 2016
Received in revised form 19 January 2017
Accepted 7 February 2017
Available online 11 February 2017

Keywords:
Salmonella proteomics
Oxidative stress
Antioxidant defense
Iron metabolism
Bacterial virulence

ABSTRACT

Salmonella Typhimurium is a bacterial pathogen that can cause widespread gastroenteritis. *Salmonella* encounters reactive oxygen species both under free-living conditions and within their mammalian host during infection. To study its response to oxidative stress, we performed the first large-scale proteomic profiling of *Salmonella* upon exposure to H_2O_2. Among 1600 detected proteins, 83 proteins showed significantly altered abundance. Interestingly, only a subset of known antioxidants was induced, likely due to distinct regulatory mechanisms. In addition, we found elevation of several *Salmonella* acquired phage products with potential contribution to DNA repair under oxidative stress. Furthermore, we observed robust induction of iron-uptake systems and disruption of these pathways led to bacterial survival defects under H_2O_2 challenge. Importantly, this work is the first to report that oxidative stress severely repressed the *Salmonella* type III secretion system (T3SS), reducing its virulence.

Biological significance

Salmonella, a Gram-negative bacterial pathogen, encounters reactive oxygen species (ROS) both endogenously and exogenously. To better understand its response to oxidative stress, we performed the first large-scale profiling of *Salmonella* protein expression upon H_2O_2 treatment. Among 1600 quantified proteins, the abundance of 116 proteins was altered significantly. Notably, iron acquisition systems were induced to promote bacterial survival under oxidative stress. Furthermore, we are the first to report that oxidative stress severely repressed *Salmonella* type III secretion system and hence reduced its virulence. We believe that these findings will not only help us better understand the molecular mechanisms that *Salmonella* has evolved to counteract ROS but also the global impact of oxidative stress on bacterial physiology.

1. Introduction

Salmonella enterica serovar Typhimurium is a gram-negative, facultative intracellular bacterial pathogen that causes acute gastroenteritis, which is characterized by inflammatory diarrhea [1,2]. *Salmonella* uses the type III secretion systems (T3SS) to interact with host cells. There are two functionally distinct T3SSs that are encoded on *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2). The T3SS apparatus is a needle-like structure that spans the inner and outer membranes of the bacterial envelope and mediates the translocation of effector proteins into host cells. These effectors have the capacity to modulate various host cell functions [3,4].

To survive in an aerobic environment, bacteria including *Salmonella* have to cope with reactive oxygen species (ROS) that are constantly generated from the respiratory chain and/or many redox enzymes. These ROS can cause widespread damage to DNA, proteins and lipids. For instance, H_2O_2 can react with ferrous iron to yield highly reactive hydroxyl radicals through the Fenton reaction [5]. DNA is a frequent target of Fenton-mediated attack due to the presence of ferrous iron in its backbone [6]. Previous studies have shown that SoxR and OxyR regulate a wide variety of oxidative responses [5]. For example, superoxide dismutase is transcriptionally regulated by SoxR. OxyR regulates the expression of both hydroperoxidase (KatG) and alkylhydroperoxidase (AhpCF), which both help bacteria degrade excessive H_2O_2.

In addition to endogenous sources, *Salmonella* also encounters exogenous oxidative stress during infection of mammalian host cells [7]. Once engulfed by professional phagocytes such as macrophages, *Salmonella* has to cope with superoxide produced by host NADPH-oxidase [8]. Rather than those detoxifying enzymes described above, most antioxidant defenses of intracellular *Salmonella* (i.e., within macrophages) are mediated by SPI-2-dependent mechanisms that prevent the colocalization of the host NADPH oxidase with the *Salmonella*-containing vacuole (SCV) [9]. Although controversy over the role of SPI-2 in

* Corresponding author at: College of Chemistry and Molecular Engineering, Peking University, 202 Chengfu Rd., Haidian District, Beijing 100871, China.
E-mail address: xiaoyun.liu@pku.edu.cn (X. Liu).
[1] These authors contributed equally to this article.

http://dx.doi.org/10.1016/j.jprot.2017.02.004
1874-3919/

ROS evasion has been raised [10], van der Heijden et al. recently described an approach that directly measures oxidative stress in *Salmonella* inside macrophages and provided convincing evidence supporting SPI-2 involvement in bacterial anti-oxidative strategies [11].

To better understand the molecular mechanisms that bacteria have evolved to counteract ROS (both endogenously and exogenously) as well as the global impact of oxidative stress on bacterial physiology, herein we conducted the first comprehensive profiling of *Salmonella* protein expression upon exposure to H_2O_2 by liquid chromatography-tandem mass spectrometry (LC-MS/MS). With detection of approximately 1600 bacterial proteins, we found 116 differentially expressed proteins. Other than induction of known antioxidants, we found evidence of extensive coordination between iron metabolism and oxidative stress. Importantly, *Salmonella* strains deficient in major iron-uptake pathways exhibited decreased survival under H_2O_2 challenge. Furthermore, we are the first to report severe inhibition of *Salmonella* SPI-1-encoded T3SS (and bacterial virulence) under oxidative stress.

2. Materials and methods

2.1. Bacterial strains, cell lines and growth conditions

The *Salmonella enterica* serovar Typhimurium wild-type strain SL1344, Δ*entC* and Δ*entC*Δ*sitBCD* mutant strains have been described previously [12]. All strains were maintained frozen at −80 °C in a peptone solution containing 5% glycerol and 2% peptone. The frozen bacteria were streaked and grown overnight on Luria-Bertani (LB) plates (15% agar and 30 μg/mL streptomycin) at 37 °C. A single colony was picked and then inoculated into 3 mL LB broth. The next morning, 150 μL of the overnight culture was inoculated into 3 mL broth. Then, H_2O_2 was added to a final concentration of 2 mM when OD_{600} (optical density at a wavelength of 600 nm) reached approximately 0.5. In approximately 2 h, the bacteria were harvested at $OD_{600} = 0.9$. Three independent biological replicates were prepared. HeLa cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies) under an atmosphere of 5% CO_2 at 37 °C.

2.2. Preparation of proteomic samples and LC-MS/MS analyses

Pelleted bacteria were resuspended in the SDS sample buffer and boiled for 5 min. Upon centrifugation at 12000 ×*g* for 2 min, the supernatant was collected and fractionated by SDS-PAGE. Subsequently, the stained gel was excised into 8 slices per sample, which were further cut into 1 mm^3 cubes. Protein in-gel digestion with trypsin was performed as described previously [13]. The resulting peptides were dried in a speedvac and resuspended in 100 mM triethyl ammonium bicarbonate (TEAB). Stable isotope dimethyl labeling was performed as reported previously [14]. Briefly, upon addition of all the samples were added with 0.6 M $NaBH_3CN$ peptide samples from bacteria treated with or without 2 mM H_2O_2 were labeled with either 4% CH_2O or CD_2O, respectively. Two forward labeling and one reverse labeling were performed in three biological replicates. After incubation for 1 h at room temperature, the labeling reaction was quenched with 1% (vol/vol) ammonia solution then 5% formic acid was added. The differentially labeled samples were mixed gently and then dried by vacuum centrifugation. The samples were stored at −80 °C prior to further LC-MS/MS analysis. Nanoflow reversed-phase LC separation was carried out on an EASY-nLC 1200 System (Thermo Scientific). The 75 μm × 150 mm capillary column was packed with 5 μm, 100 Å Magic C18AQ silica-based particles (Michrom BioResources Inc., Auburn, CA). The column had an integrated electrospray tip that was made in-house with a laser-based puller (Model P-2000, Sutter Instruments). The mobile phase consisted of solvent A (97% H_2O, 3% acetonitrile, and 0.1% formic acid) and solvent B (80% acetonitrile, 20% H_2O and 0.1% formic acid). The LC gradient started at 7% solvent B for 3 min and then was linearly increased to 40% over 60 min. Next, the gradient was quickly ramped to 90% in 2 min and stayed there for 10 min. The gradient was then switched back to 100% solvent A for column equilibration. The flow rate was kept at 300 nL/min. The MS and MS/MS analyses were performed on a hybrid linear ion trap – Orbitrap mass spectrometer (Orbitrap Velos, Thermo Scientific). A full MS scan (*m*/*z* range 350–1500) was followed by MS/MS analyses of the ten most abundant ions. Dynamic exclusion was enabled with a repeat duration of 15 s and exclusion duration of 15 s. With three pairs of biological replicates (control and H_2O_2-treated samples) and 8 gel fractions per sample, we carried out 24 LC-MS/MS experiments (3 × 8).

For preparation of the *Salmonella* secretome, bacterial subculture ($OD_{600} = 0.9$) was centrifuged at 10,000 ×*g* for 5 min and the culture supernatant containing secreted T3SS effectors was collected and passed through a 0.22 μm membrane filter. Trichloroacetic acid (TCA) precipitation was then carried out at 4 °C overnight and protein pellets were collected after centrifugation at 15,000 ×*g* for 15 min at 4 °C and washed twice with cold acetone [15]. The precipitates were prepared for LC-MS analyses as described above.

2.3. Proteomic data processing

For data analyses, raw LC-MS files were searched against a non-redundant S. Typhimurium LT2 protein database (www.uniprot.org) by using MaxQuant 1.5.3.30 [16]. Peptide N-term dimethylation and lysine dimethylation were chosen for relative protein quantification. Precursor mass tolerance of peptides was set as 4.5 ppm, and for fragment ions in MS/MS scans the mass tolerance was set as 0.8 Da. Cysteine carbamidomethylation was set as a fixed modification and methionine oxidation was set as a variable modification. A maximum of two missed cleavage sites were allowed. The peptide and protein identifications were filtered at false discovery rates <1%. Raw H/L ratios were further processed by Perseus 1.5.4.1 [17]. Proteins that have quantification ratios in at least two replicates were considered quantified. Missing values in H/L ratios were replaced by average ratios of the other two replicates. We considered proteins with significant differences when the average fold change was ≥2 or ≤0.5, and *p*-value was <0.05 (paired Student's *t*-test using the data analysis tools of Microsoft Excel). For secretome analyses, the label-free quantitation (LFQ) intensity for each protein was calculated by using MaxQuant.

2.4. ICP-MS measurements of iron levels

A triple quadrupole inductively coupled plasma mass spectrometer (ICP-MS, Agilent 8800) was used to determine iron levels. The LB media with and without the addition of 2 mM H_2O_2 were centrifuged at 20,000 ×*g* for 10 min. Then, the supernatant was collected and processed for ICP-MS analyses. The MS instrument was operated in O_2 reaction mode.

2.5. Construction of Salmonella mutant strains

Salmonella Δ*entC* and Δ*entC*Δ*sitBCD* double mutant strains were constructed using the standard homologous recombination method as described previously[12]. Briefly, a PCR fragment containing two flanking sequences of the *entC* gene was first cloned into a suicide plasmid, pSR47s, which was subsequently transferred into the WT strain to construct the Δ*entC* mutant through *E. coli* DH5α (λpir)-mediated conjugation. LB agar plates containing 50 μg/mL kanamycin and 30 μg/mL streptomycin were used to screen for potentially successful transconjugants. Then, those colonies were further screened on LB agar plates containing 15% sucrose without NaCl for markerless in-frame deletion. The successful Δ*entC* mutants were finally confirmed by PCR and sequencing. The Δ*entC*Δ*sitBCD* double mutant strain was constructed as described above. A list of all oligonucleotide primers was provided as Supplemental Table S2.

2.6. H_2O_2 survival assay

For the three strains (WT, Δ*entC* and Δ*entC*Δ*sitBCD*) to be examined in an H_2O_2 survival assay, overnight bacterial cultures were diluted 1:20 into 4 mL LB media. Bacterial cells were treated with a final concentration of 10 mM H_2O_2 at OD_{600} of 0.45 for 30 min (for proteome measurements, 2 mM H_2O_2 was used). Then, the viable bacteria were enumerated by counting the number of colony-forming units (CFU). Percent survival was calculated as the ratio of viable bacteria counts after and before H_2O_2 treatment.

2.7. Salmonella invasion assay

A *Salmonella* invasion assay was performed as described before [12]. Briefly, HeLa cells were seeded at 5×10^5 cells per well in a 6-well plate one day before infection. Infection was carried out in Hank's Balanced Salt Solution (HBSS) for 30 min with a multiplicity of infection (MOI) of 10. For stimulation by oxidative stress, bacteria samples were cultured in LB broth with the addition of 2 mM H_2O_2 for ~2 h prior to infection. After infection, cell monolayers were washed twice with preheated HBSS and further incubated in fresh DMEM containing 100 μg/mL gentamicin for 1 h to kill extracellular bacteria. Then, the cell monolayers were washed with PBS twice and lysed in a 20 mM Tris-HCl (pH = 7.6) buffer containing 150 mM NaCl and 1% Triton X-100. The internalized bacteria were quantified by plating serial dilutions onto LB agar plates. The percent invasion rate was calculated as the ratio of internalized bacteria and the inoculum.

2.8. Quantitative real time PCR (qRT-PCR) analyses

Total bacterial RNA was extracted after 2 mM H_2O_2 treatment for 2 h. RNA was isolated with the EasyPure RNA kit (TransGen Biotech) according to the manufacturer's instructions. The resulting RNA samples were used to synthesize first-strand cDNA with EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix (Transgen Biotech). RT-PCR was performed by using UltraSYBR Mixture (Low ROX) (CWBIO). The PCR primers used for qRT-PCR assays are listed in Supplemental Table S2. The relative expression levels of each gene were calculated by the $2^{-\Delta\Delta CT}$ method [18] with the 16S rRNA gene as the internal control. Triplicate analyses were performed with RNA samples harvested from three independent experiments.

2.9. Validation of proteomic data by Western blotting analyses

The 3×FLAG-tagged *Salmonella* strains were generated by the λRed method [19]. Upon 2 mM H_2O_2 treatment for 2 h, bacterial proteins were first run on SDS-PAGE and further transferred to polyvinylidene difluoride (PVDF) membranes. Subsequent immunoblotting was performed with primary antibodies specific for *Salmonella* DnaK (Enzo Life Sciences) (1:5000) or FLAG (Cwbio, China) (1:2000) and horseradish peroxidase (HRP)-conjugated secondary antibodies (Cwbio, China) (1:5000).

3. Results

3.1. Global proteomic survey of S. Typhimurium in response to H_2O_2 treatment

To study differential protein expression of *S.* Typhimurium under oxidative stress, we added 2 mM H_2O_2 to bacterial culture and compared the proteome to that of non-treated controls. In previous studies, 1–5 mM H_2O_2 was typically applied to bacteria for different durations [20–22]. Compared to controls, we found indistinguishable *Salmonella* growth rate upon 1 mM H_2O_2 treatment (Fig. 1A), while at 2 mM H_2O_2 we detected robust induction of most antioxidant pathways with minimal growth inhibition. More pronounced growth inhibition

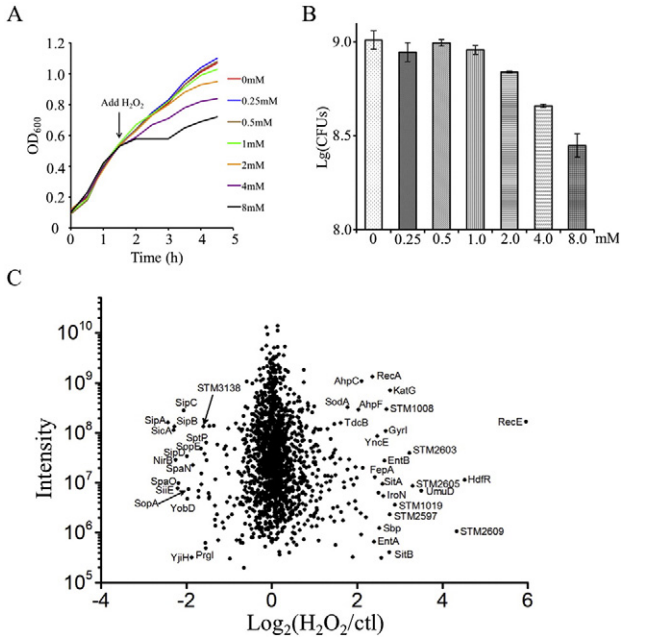

Fig. 1. (A) Growth curves of wild-type (WT) *Salmonella* with different concentrations of H_2O_2 added to the LB media when OD_{600} reached 0.5. (B) CFU assays of wild-type (WT) *Salmonella* ($OD_{600} = 0.5$) upon treatment with different concentrations of H_2O_2 for 2 h. (C) Scatter plot of *S.* Typhimurium protein fold-difference versus individual protein abundance. The fold ratio was calculated by dividing ion intensities in H_2O_2-treated samples by those of the controls. The average fold changes are reported on the x-axis. The y-axis plots ion intensities of a given protein that were obtained from MaxQuant. The proteins appearing to the left or the right indicate down- or up- regulation, respectively.

was observed with H_2O_2 levels >2 mM. Additional data from CFU assays correlate well with bacterial growth at various H_2O_2 levels (Fig. 1B). In total, we detected 1666 *Salmonella* proteins from three biological replicates, with approximately 1566 identifications per bacterial sample. We found 48 up-regulated proteins and 35 down-regulated proteins (Table S1). When protein fold differences were graphed as a function of their relative abundance (denoted by ion intensity values) (Fig. 1C), notably up-regulated proteins exhibited greater fold changes in comparison to down-regulated ones. In particular, a number of proteins showed >5-fold increases in their abundance. In addition to known antioxidants and many components of DNA repair machinery, remarkably, we observed drastic elevation of *Salmonella* iron-acquisition pathways. Intriguingly, the largest group of down-regulated proteins is mostly comprised of bacterial virulence factors.

3.2. Known antioxidant proteins and DNA repair machinery were robustly induced under H_2O_2 stress

Fig. 2 shows an overview of the major *Salmonella* antioxidant pathways that were measured in this study. We detected all *Salmonella* H_2O_2-degrading enzymes, including three catalases (KatE, KatG and KatN) and three peroxidases (AhpCF, TsaA and Tpx). Our data indicate that detoxifying enzymes KatG and AhpCF exhibited substantially higher levels upon treatment while KatE and KatN were not induced. In addition to AhpCF, *Salmonella* abundantly expressed two other peroxidases, TsaA and Tpx, though they were not significantly altered. In agreement with our data, the *E. coli ahpC* transcript was highly induced in response to oxidative stress, while *tpx* was not [23]. Interestingly, expression of various superoxide dismutases exhibit different trends with a much higher level of SodA and slightly lower/unchanged levels of SodB and SodC. In addition to direct ROS detoxification, *Salmonella* has evolved mechanisms to repair H_2O_2-caused cellular damage, such as DNA lesions. In total, we found six up-regulated proteins being essential components of DNA repair machinery (e.g., RecA, RecE, and GyrI).

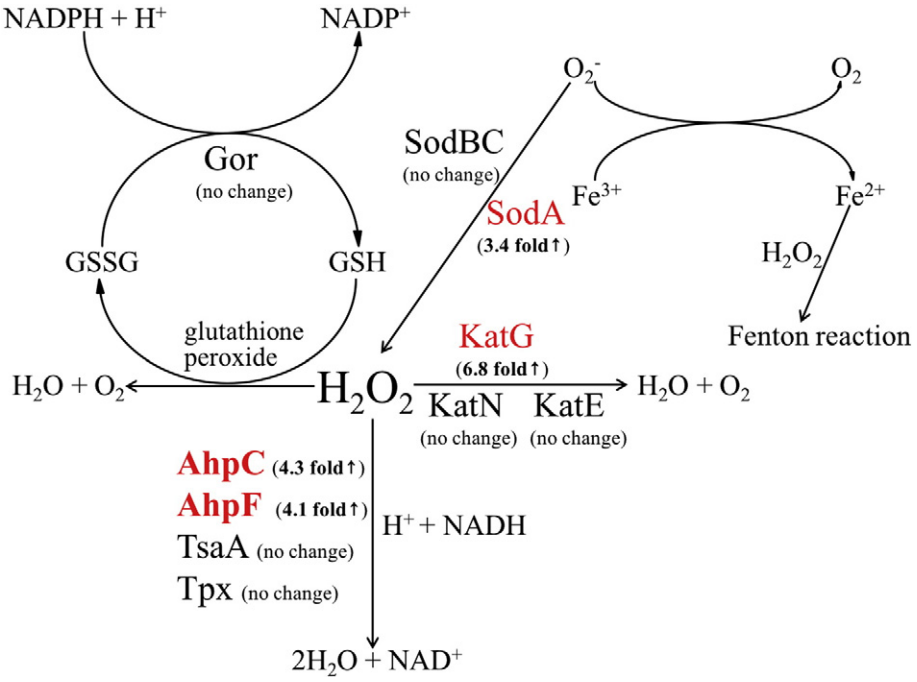

Fig. 2. The main *Salmonella* antioxidant pathways that were detected in this study. Observed enzymes with antioxidant activities were color-coded with red, green and black, denoting up-regulation, down-regulation and no significant changes, respectively. The fold difference is displayed in brackets.

3.3. Some Salmonella prophage proteins were induced under oxidative stress

In this study two of highly elevated proteins, STM2603 and STM1008, are encoded in the Gifsy-1 and Gifsy-2 prophage gene clusters respectively (Table S1). As lysogenic phage induction under UV or H_2O_2 exposure was noted years ago [24], next we sought to determine if elevation of these two proteins was due to general phage induction (i.e., more phage assemblies). By using qPCR interestingly we found no significant increase in the DNA copies of STM2603 and STM1008 upon H_2O_2 treatment (Fig. 3A). To further determine whether the expression of those prophage proteins was regulated on the transcriptional level, next we measured their mRNA levels by using quantitative RT-PCR (qRT-PCR). Upon 2 mM H_2O_2 treatment, the transcript levels of STM1008 and STM2603 were increased 2–6 times (Fig. 3B), suggesting their elevated protein expression was, at least in part, regulated on the transcriptional level. In addition, we also measured the transcript levels of three prophage genes (STM1005, STM1006 and STM2607) in the same operons as STM1008 and STM2603 (Fig. 3B). We found that not all genes in these operons underwent significant changes on the transcription (e.g., STM1005 was unaltered). It's also interesting to note that *Salmonella* encodes another Gifsy-2 prophage protein, STM2633, with almost the exact same sequence as STM1008, and the neighboring proteins (STM2632/STM1009) are also duplicates. We found that both STM1008 and STM2633 contain a RecT domain, which is implicated in the DNA recombination pathway [25]. Taken together, these results indicate selective induction of some prophage proteins on both transcript and protein levels. More importantly, rather than general phage induction, such regulation is more likely to be driven by *Salmonella* responses to DNA damage under oxidative stress.

3.4. Salmonella iron acquisition systems were induced to promote bacterial survival and/or growth under H_2O_2 stress

A large class of up-regulated proteins is involved in iron acquisition and utilization pathways (Fig. 4A). Siderophore-mediated iron uptake is one of the most common mechanisms for bacterial iron acquisition and *Salmonella* can synthesize and secrete two types of siderophores,

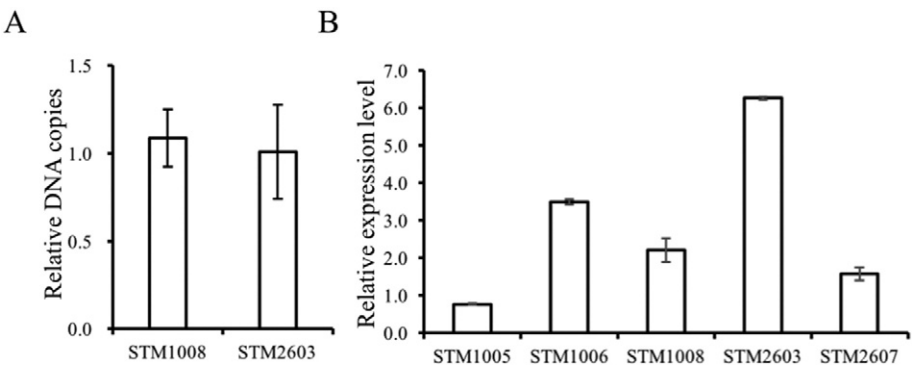

Fig. 3. (A) Quantitative PCR analysis of prophage genes STM1008 and STM2603. The relative DNA copies of prophage genes STM1008 and STM2603 without H_2O_2 treatment were arbitrarily set to 1. (B) Quantitative RT-PCR analyses for prophage genes. The relative expression of prophage genes without oxidative stress was arbitrarily set to 1.

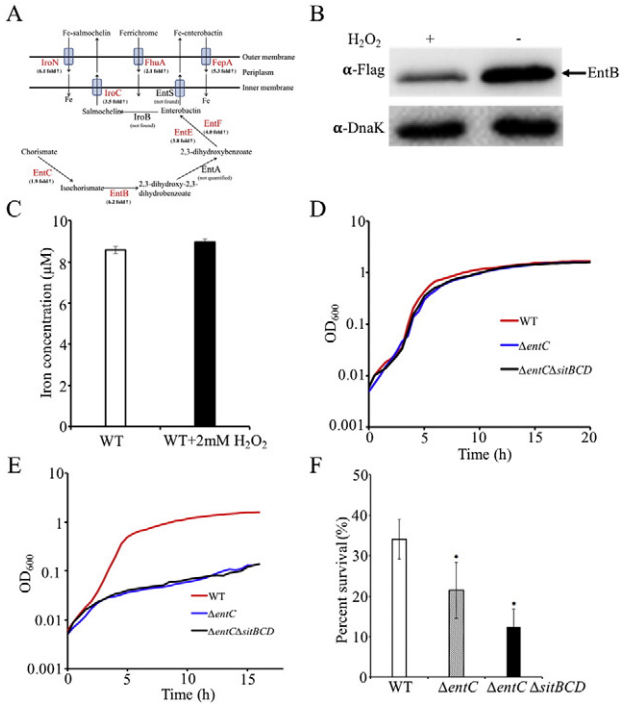

Fig. 4. (A) The *Salmonella* iron uptake and siderophore biosynthesis pathways. The fold increase of up-regulated proteins is displayed in brackets. (B) Immunoblotting analyses of EntB expression upon H_2O_2 treatment. (C) The iron levels in LB media, with and without H_2O_2 addition. (D) Growth curves of wild-type (WT), Δ*entC* and Δ*entC*Δ*sitBCD* strains in LB broth. (E) Growth curves of wild-type (WT), Δ*entC* and Δ*entC*Δ*sitBCD* strains in iron-deficient LB broth (with 280 μM 2,2′-dipyridine (DIP)). (F) H_2O_2 survival assay. WT, Δ*entC* and Δ*entC*Δ*sitBCD* strains were exposed to a final concentration of 10 mM H_2O_2 for 30 min and viable bacteria were numerated by the colony forming unit (CFU) assay. Results from three independent measurements are plotted. * indicates $P < 0.05$ (one-way ANOVA using the data analysis functions of Microsoft Excel).

enterobactin and salmochelin [26]. Enterobactin biosynthesis proteins (EntB, EntC, EntE and EntF) were up-regulated (approximately 2-fold to 6-fold) except EntA (not quantified) and EntD (not detected). In agreement with our proteomic data, immunoblotting analysis of 3× Flag-tagged EntB also showed increased protein abundance upon peroxide treatment (Fig. 4B). IroC mediates salmochelin translocation through the inner membrane, and its abundance increased by 3.5-fold. Importantly, many outer membrane receptors were also up-regulated, including IroN (6.1-fold), FepA (5.3-fold) and FhuA (2.1-fold). In addition, the Suf system (SufBCDS) was also highly induced (2.1-fold to 4.2-fold). These Suf proteins assist in Fe—S cluster assembly and/or repair ROS-caused damage [27]. Consistent with our findings, *Salmonella suf* transcripts were also strongly induced upon peroxide treatment, though under SPI-2 inducing conditions [21].

Up-regulation of iron uptake can be triggered by iron deficiency, either extracellularly or intracellularly. To differentiate these possibilities, we next measured iron levels in LB media by using inductively coupled plasma mass spectrometry (ICP-MS) (Fig. 4C). We found that H_2O_2 addition did not reduce the level of iron due to potential precipitation or other differences that may be incurred. Therefore, the induction of iron acquisition pathways is more likely due to intracellular iron starvation. To further test the importance of iron uptake under peroxide stress, next we constructed *Salmonella* strains deficient in these systems (Δ*entC* and Δ*entC*Δ*sitBCD*). Relative to wild-type bacteria, both mutants grew comparably well in LB media while exhibited severe growth defects under iron-limiting conditions (Fig. 4D and E). When challenged by high levels of H_2O_2 (10 mM), *Salmonella* Δ*entC* strains exhibited significantly reduced survival and the double mutant (Δ*entC*Δ*sitBCD*) showed an even more severe defect (Fig. 4F). Taken together, these findings support a vital role of *Salmonella* iron acquisition in promoting bacterial survival under oxidative stress.

3.5. Expression of *Salmonella* SPI-1 T3SS was significantly inhibited under H_2O_2 stress

Among down-regulated proteins, *Salmonella* SPI-1-encoded virulence factors comprise one of the largest groups (25%, shown in Table 1) and many of them are among the most repressed proteins. Further classification reveals that nearly half of them are structural components of the secretion apparatus. For example, translocon proteins (SipB, SipC and SipD) were approximately 4× less abundant under oxidative stress. As a component of the export apparatus [28], SpaO was 4.5-fold less abundant. Other apparatus components such as PrgH, InvH and OrgA were also repressed (2–3× less). Notably, many effector proteins were down-regulated as well including SopA, SopE/E2, SipA, and SptP. To further verify our proteomic data, we constructed *Salmonella* strains chromosomally expressing 3×Flag-tagged SptP and subsequent Western blotting analysis revealed down-regulation of this effector protein as well (Fig. 5A).

Next we sought to ask if such global repression of SPI-1 T3SS occurs at the transcriptional level. Interestingly, several known SPI-1 regulators were down-regulated (e. g., HilA was 2.8-fold less abundant). Next we aimed to determine if restoration of HilA expression can rescue the inhibition of SPI-1 T3SS under H_2O_2 stress. We constructed a *Salmonella*

Table 1
Functional classifications of *Salmonella* T3SS virulence factors that were down-regulated.

Functional class	Gene	Protein	Fold	*p*-Value
Translocon	*sipB*	Cell invasion protein SipB	−4.9	<0.001
	sipC	Cell invasion protein SipC	−4.2	0.001
	sipD	Cell invasion protein SipD	−4.0	0.013
Structural components	*prgH*	Protein PrgH	−2.4	0.020
	prgK	Lipoprotein PrgK	−1.5	0.044
	invH	Invasion protein InvH	−3.0	0.001
	orgA	Oxygen-regulated invasion protein OrgA	−3.3	<0.001
	orgB	Oxygen-regulated invasion protein OrgB	−3.1	0.001
	spaO	Protein SpaO	−4.5	<0.001
Effectors	*sopA*	Secreted effector protein of *Salmonella*	−3.9	0.002
	sopB	Inositol phosphate phosphatase SopB	−2.0	0.058
	sopE	Guanine nucleotide exchange factor SopE	−3.2	0.003
	sopE2	Guanine nucleotide exchange factor SopE2	−3.3	0.013
	sipA	Cell invasion protein SipA	−5.4	<0.001
	sptP	Effector protein SptP	−2.9	0.004
	slrP	Leucine-rich repeat protein	−1.7	0.043
Transcription factors	*hilA*	Transcriptional regulator HilA	−2.9	0.015
	hilD	Transcriptional regulator HilD	−1.8	0.034
Chaperones	*sicA*	Chaperone protein SicA	−4.9	<0.001

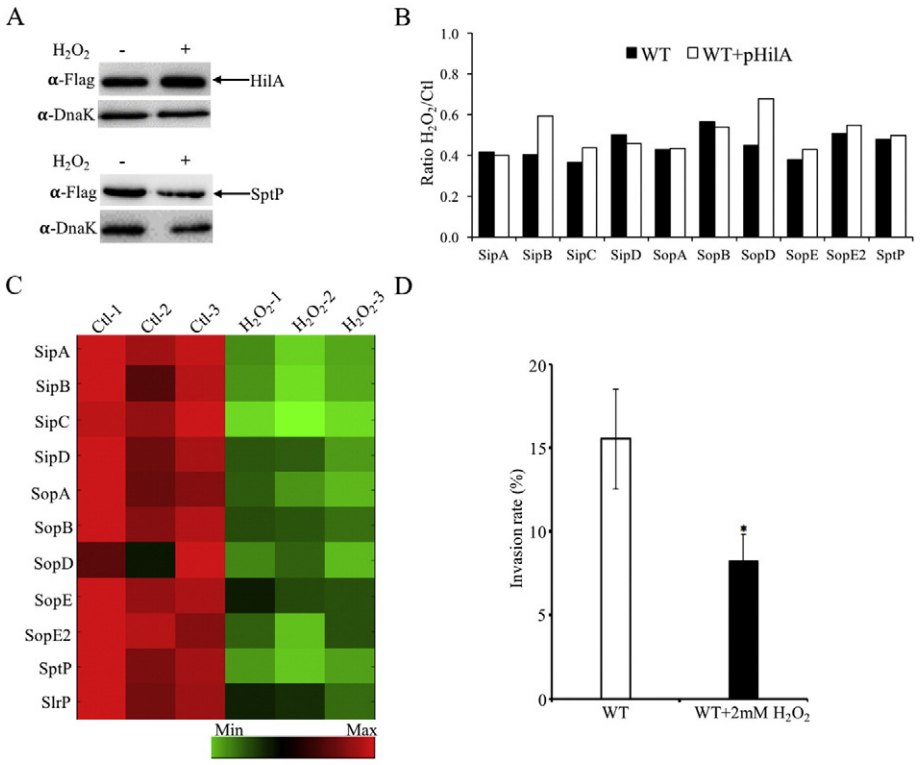

Fig. 5. (A) The upper panels: Western blotting analyses of *Salmonella* strains constitutively expressing HilA before and after H_2O_2 treatment. HilA-3×Flag expression plasmids were introduced into wild-type *Salmonella* strain SL1344. The lower panels: Western blotting analyses of *Salmonella* strains expressing 3×Flag-tagged SptP before and after H_2O_2 treatment. (B) Fold changes of expression of SPI-1 T3SS effectors under oxidative stress with (solid) and without (blank) constitutively expressed HilA. (C) Heat maps showing the abundance levels of *Salmonella* virulence factors that were identified in bacterial culture supernatant. Three biological replicates were analyzed with Ctl-1, Ctl-2 and Ctl-3 denoting control samples, and H_2O_2-1, H_2O_2-2 and H_2O_2-3 corresponding to H_2O_2-treated samples. (D) *Salmonella* invasion assay. Bacteria with and without 2 mM H_2O_2 treatment for 2 h were allowed to infect HeLa cells for 30 min. Percent invasion from four independent measurements is plotted. * indicates $P < 0.05$ (Student's *t*-test using the data analysis functions of Microsoft Excel).

strain in which HilA is constitutively expressed from a plasmid. As shown in Fig. 5A, its abundance level was not reduced upon 2 mM H_2O_2 treatment. However, steady expression levels of HilA failed to rescue the inhibition of SPI-1 T3SS expression under peroxide stress (Fig. 5B). In addition, we also found that the constitutive expression of HilD, an AraC-like activator of HilA [29], did not rescue the down-regulation of SPI-1 T3SS effectors either (data not shown). Together, these data suggest that the general repression of SPI-1 T3SS is unlikely to be transcriptionally regulated (i.e., HilA and HilD independent at least).

We reasoned that SPI-1 repression likely results in lower levels of secretion as well. Next we measured the *Salmonella* secretome by using quantitative LC-MS. Indeed we found substantially lower amounts of most effectors (and the translocon components) in the bacterial culture supernatant (Fig. 5C and Table S1). Considering the critical role of SPI-1 T3SS in mediating bacterial invasion, next we sought to assess the impact of oxidative stress on *Salmonella* internalization into host epithelial cells. Consistently, we found that peroxide exposure markedly reduced bacterial entry into HeLa cells (approximately 50% less, Fig. 5D). Taken together, these data indicate the strong inhibition of *Salmonella* SPI-1 T3SS under oxidative stress.

4. Discussion

Thus far, most studies of *Salmonella* (or closely related *E. coli*) oxidative responses have focused on either a handful proteins or global transcriptome analyses [20,21,30–33].For example, Zheng et al. examined the transcriptional profiles of *E. coli* wild-type and *oxyR* deletion strains upon H_2O_2 treatment [31]. Lately, Kröger et al. reported RNA-seq-based transcriptomic analyses of *Salmonella* in response to H_2O_2 under SPI-2 inducing conditions [21]. As mRNA data are sometime insufficient to predict protein abundance due to post-transcriptional regulation [34], global profiling of *Salmonella* proteome under oxidative conditions is desirable. A while ago, such work was carried out on *Salmonella enterica* serovar Enteritidis via 2D gel electrophoresis and MALDI mass spectrometry [22], in which the expression profiles of 103 bacterial proteins were reported.

With >1600 total protein identifications, our study significantly expands the proteome coverage under oxidative stress. In comparison with previous transcriptome data, many known antioxidant genes, such as *katG* and *ahpCF*, were regulated similarly at both the transcript and protein levels. However, for several highly induced *E. coli* genes (*dpS*, *yfiA*, *grxA*, and *jbpB*) at the transcript level [31], the corresponding *Salmonella* proteins were not altered in our study. Importantly, under our experimental conditions many known antioxidants were not induced, including KatE, KatN, TsaA, Tpx, SodB and SodC, indicative of different regulatory mechanisms. Unlike OxyR-regulated KatG and AhpCF, the transcription of KatE and KatN was thought to be controlled by RpoS, a regulator of gene products important for bacterial growth at the stationary phase [35]. Consistent with our findings, Loewen et al. reported that *E. coli* KatG was most produced in the logarithmic phase and highly induced by H_2O_2, but KatE was not [36]. Furthermore, it was shown that *katN* mutation did not affect the ability of *Salmonella* to resist H_2O_2 shock in the exponential phase [35]. Interestingly, we found substantially different expression levels of these common antioxidants with TsaA being the most abundant one. Though not induced under H_2O_2 shock, this protein may play an important role in bacterial oxidative defense.

Furthermore, we found elevation of the superoxide dismutase [Mn] SodA, whereas the level of iron-dependent SodB was slightly decreased, likely due to iron starvation. Conceivably, induction of Mn-dependent SodA might help mitigate the partial loss of SodB activity due to insufficient iron [37,38]. Additionally, we observed induction of many proteins associated with iron uptake. Increased iron uptake seems to contradict minimizing iron toxicity at the first glance; however, sufficient iron

supply is required to sustain bacterial growth so these opposing requirements likely must be carefully balanced. Next we further demonstrated that iron uptake contributes to bacterial survival under oxidative stress because *Salmonella* strains deficient in siderophore-based pathways and the Sit system exhibited severe survival defects under high levels of H_2O_2 challenge. Consistently, a previous study reported that the Sit operon, in combination with MntH, may mediate bacterial resistance to H_2O_2 [39].

Additionally, we are the first to report significant repression of *Salmonella* SPI-1 T3SS under oxidative stress. Previously, Kim et al. found opposing changes of two virulence factors SipC and SopB upon exposure to H_2O_2 [22], though they used a different strain (*Salmonella* enterica serovar Enteritid SE2472a) that may account for the discrepancies. Our data reveal unanimous down-regulation of SPI-1 proteins encompassing structural components, effectors and transcriptional regulators. Previously, Frye et al. reported lower transcript levels of SPI-1 genes *invA-J*, *sipA-D*, and *prgHIJK* in *Salmonella* LT2 strain under oxidative stress, which seem to agree well with our proteome data (Table1). Interestingly, under oxidative stress transcription of SPI-2 T3SS was also inhibited [21]. However, distinct bacterial culturing conditions (SPI-2 inducing vs SPI-1 inducing) preclude direct comparison between the RNA-seq and our proteomic data. In fact, RNA-seq analyses showed rather low or largely unaltered levels of most SPI-1 transcripts. Furthermore, we explored the possibility that any known SPI-1 regulator may be involved in the global suppression of SPI-1 proteins under oxidative stress. Constitutive expression of such regulators, HilA and HilD, failed to restore the expression levels of most SPI-1-encoded proteins, implicating a post-transcriptional mechanism for SPI-1 inhibition upon H_2O_2 treatment. Moreover, *Salmonella* secretome analyses found significantly lower amounts of SPI-1 effectors in the bacterial culture supernatant, suggesting reduced virulence upon H_2O_2 treatment. Consistent with this notion, *Salmonella* invasion assays demonstrated much lower rates of bacterial internalization during infection of host epithelial cells.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2017.02.004.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

We are grateful to the members of the Liu laboratory for their careful review of this manuscript. This work was supported by grants from the National Natural Science Foundation of China (21622501, 21475005) and the Thousand Young Talents program of the Chinese government.

References

[1] K.H. Darwin, V.L. Miller, Molecular basis of the interaction of *Salmonella* with the intestinal mucosa, Clin. Microbiol. Rev. 12 (1999) 405–428.
[2] M.E. Ohl, S.I. Miller, *Salmonella*: a model for bacterial pathogenesis, Annu. Rev. Med. 52 (2001) 259–274.
[3] S.R. Waterman, D.W. Holden, Functions and effectors of the *Salmonella* pathogenicity island 2 type III secretion system, Cell. Microbiol. 5 (2003) 501–511.
[4] J.E. Galán, M. Lara-Tejero, T.C. Marlovits, S. Wagner, Bacterial Type III secretion systems: specialized nanomachines for protein delivery into target cells, Annu. Rev. Microbiol. 68 (2014) 415–438.
[5] J.A. Imlay, Pathways of oxidative damage, Annu. Rev. Microbiol. 57 (2003) 395–418.
[6] J.A. Imlay, S. Linn, DNA damage and oxygen radical toxicity, Science 240 (1988) 1302–1309.
[7] J.A. Imlay, The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium, Nat. Rev. Microbiol. 11 (2013) 443–454.
[8] R. Janssen, T. van der Straaten, A. van Diepen, J.T. van Dissel, Responses to reactive oxygen intermediates and virulence of *Salmonella* Typhimurium, Microbes Infect. 5 (2003) 527–534.
[9] A. Vazquez-Torres, Y.S. Xu, J. Jones-Carson, D.W. Holden, S.M. Lucia, M.C. Dinauer, P. Mastroeni, F.C. Fang, *Salmonella* pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase, Science 287 (2000) 1655–1658.
[10] L. Aussel, W. Zhao, M. Hébrard, A.A. Guilhon, J.P. Viala, S. Henri, L. Chasson, J.P. Gorvel, F. Barras, S. Méresse, *Salmonella* detoxifying enzymes are sufficient to cope with the host oxidative burst, Mol. Microbiol. 80 (2011) 628–640.
[11] J. van der Heijden, E.S. Bosman, L.A. Reynolds, B.B. Finlay, Direct measurement of oxidative and nitrosative stress dynamics in *Salmonella* inside macrophages, Proc. Natl. Acad. Sci. U. S. A. 112 (2015) 560–565.
[12] Y. Liu, Q. Zhang, M. Hu, K. Yu, J. Fu, F. Zhou, X. Liu, Proteomic analyses of intracellular *Salmonella enterica* serovar Typhimurium reveal extensive bacterial adaptations to infected host epithelial cells, Infect. Immun. 83 (2015) 2897–2906.
[13] M. Hu, Y. Liu, K. Yu, X. Liu, Decreasing the amount of trypsin in in-gel digestion leads to diminished chemical noise and improved protein identifications, J. Proteomics 109 (2014) 16–25.
[14] P.J. Boersema, R. Raijmakers, S. Lemeer, S. Mohammed, A.J. Heck, Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics, Nat. Protoc. 4 (2009) 484–494.
[15] A.J. Link, J. LaBaer, Trichloroacetic acid (TCA) precipitation of proteins, Cold Spring Harb. Protoc. 993–994 (2011).
[16] J. Cox, M. Mann, MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification, Nat. Biotechnol. 26 (2008) 1367–1372.
[17] S. Tyanova, T. Temu, P. Sinitcyn, A. Carlson, M.Y. Hein, T. Geiger, M. Mann, J. Cox, The Perseus computational platform for comprehensive analysis of (prote)omics data, Nat. Methods (2016) (Advance online publication).
[18] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25 (2001) 402–408.
[19] K.C. Murphy, Use of bacteriophage lambda recombination functions to promote gene replacement in *Escherichia coli*, J. Bacteriol. 180 (1998) 2063–2071.
[20] E. Morales, I.L. Calderón, B. Collao, F. Gil, S. Porwolik, M. McClelland, et al., Hypochlorous acid and hydrogen peroxide-induced negative regulation of *Salmonella enterica* serovar Typhimurium *ompW* by the response regulator ArcA, BMC Microbiol. 12 (2012) 63.
[21] C. Kröger, A. Colgan, S. Srikumar, K. Händler, S.K. Sivasankaran, D.L. Hammarlöf, et al., An infection-relevant transcriptomic compendium for *Salmonella enterica* serovar Typhimurium, Cell Host Microbe 14 (2013) 683–695.
[22] K. Kim, E. Yang, G.P. Vu, H. Gong, J. Su, F. Liu, et al., Mass spectrometry-based quantitative proteomic analysis of *Salmonella enterica* serovar Enteritidis protein expression upon exposure to hydrogen peroxide, BMC Microbiol. 10 (2010) 166.
[23] Cha Mk, W.C. Kim, C.J. Lim, K. Kim, I.H. Kim, *Escherichia coli* periplasmic thiol peroxidase acts as lipid hydroperoxide peroxidase and the principal antioxidative function during anaerobic growth, J. Biol. Chem. 279 (2004) 8769–8778.
[24] J. Baluch, R. Sussman, Correlation between UV dose requirement for lambda bacteriophage induction and lambda repressor concentration, J. Virol. 26 (1978) 595–602.
[25] L.M. Iyer, E.V. Koonin, L. Aravind, Classification and evolutionary history of the single-strand annealing proteins, RecT, Redbeta, ERF and RAD52, BMC Genomics 3–8 (2002).
[26] M.V. Crouch, M. Castor, J.E. Karlinsey, T. Kalhorn, F.C. Fang, Biosynthesis and IroC-dependent export of the siderophore salmochelin are essential for virulence of *Salmonella enterica* serovar Typhimurium, Mol. Microbiol. 67 (2008) 971–983.
[27] L. Nachin, L. Loiseau, D. Expert, F. Barras, SufC: an unorthodox cytoplasmic ABC/ATPase required for [Fe-S] biogenesis under oxidative stress, EMBO J. 22 (2003) 427–437.
[28] T.G. Kimbrough, S.I. Miller, Assembly of the type III secretion needle complex of *Salmonella* Typhimurium, Microbes Infect. 4 (2002) 75–82.
[29] L.M. Schechter, S.M. Damrauer, C.A. Lee, Two AraC/XylS family members can independently counteract the effect of repressing sequences upstream of the hilA promoter, Mol. Microbiol. 32 (1999) 629–642.
[30] T.A. Halsey, A. Vazquez-Torres, D.J. Gravdahl, F.C. Fang, S.J. Libby, The ferritin-like dps protein is required for *Salmonella enterica* serovar Typhimurium oxidative stress resistance and virulence, Infect. Immun. 72 (2004) 1155–1158.
[31] M. Zheng, X. Wang, L.J. Templeton, D.R. Smulski, R.A. LaRossa, G. Storz, DNA microarray-mediated transcriptional profiling of the *Escherichia coli* response to hydrogen peroxide, J. Bacteriol. 183 (2001) 4562–4570.
[32] M.F. Christman, R.W. Morgon, F.S. Jacobson, B.N. Ames, Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella* Typhimurium, Cell 41 (1985) 753–762.
[33] R.A. VanBogelen, P.M. Kelley, F.C. Neidhardt, Differential induction of heat shock, SOS, and oxidation stress regulons and accumulation of nucleotides in *Escherichia coli*, J. Bacteriol. 169 (1987) 26–32.
[34] S.P. Gygi, Y. Rochon, B.R. Franza, R. Aebersold, Correlation between protein and mRNA abundance in yeast, Mol. Cell. Biol. 19 (1999) 1720–1730.
[35] V. Robbe-Saule, C. Coynault, M. Ibanez-Ruiz, D. Hermant, F. Norel, Identification of a non-haem catalase in *Salmonella* and its regulation by RpoS (σs), Mol. Microbiol. 39 (2001) 1533–1545.
[36] P.C. Loewen, J. Switala, B.L. Triggs-Raine, Catalases HPI and HPII in *Escherichia coli* are induced independently, Arch. Biochem. Biophys. 243 (1985) 144–149.
[37] D. Touati, M. Jacques, B. Tardat, L. Bouchard, S. Despied, Lethal oxidative damage and mutagenesis are generated by iron in Δ*fur* mutants of *Escherichia coli*: protective role of superoxide dismutase, J. Bacteriol. 177 (1995) 2305–2314.
[38] S.B. Farr, R. D' Ari, D. Touati, Oxygen-dependent mutagenesis in *Escherichia coli* lacking superoxide dismutase, Proc. Natl. Acad. Sci. U. S. A. 83 (1986) 8268–8272.
[39] M. Sabri, S. Léveillé, C.M. Dozois, A SitABCD homologue from an avian pathogenic *Escherichia coli* strain mediates transport of iron and manganese and resistance to hydrogen peroxide, Microbiology 152 (2006) 745–758.