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Insights into the Oxidative Stress Response of *Salmonella enterica* serovar Enteritidis Revealed by the Next Generation Sequencing Approach

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Abstract: As a facultative intracellular pathogen, *Salmonella* Enteritidis must develop an effective oxidative stress response to survive exposure to reactive oxygen species within the host. To study this defense mechanism, we carried out a series of oxidative stress assays in parallel with a comparative transcriptome analyses using a next generation sequencing approach. It was shown that the expression of 45% of the genome was significantly altered upon exposure to H_2O_2. Quantitatively the most significant ($\geq$100 fold) gene expression alterations were observed among genes encoding the sulfur utilization factor of Fe-S cluster formation and iron homeostasis. Our data point out the multifaceted nature of the oxidative stress response. It includes not only numerous mechanisms of DNA and protein repair and redox homeostasis, but also the key genes associated with osmotic stress, multidrug efflux, stringent stress, decrease influx of small molecules, manganese and phosphate starvation stress responses. Importantly, this study revealed that oxidatively stressed *S.* Enteritidis cells simultaneously repressed key motility encoding genes and induced a wide range of adhesin- and salmonellae-essential virulence-encoding genes, that are critical for the biofilm formation and intracellular survival, respectively. This finding indicates a potential intrinsic link between oxidative stress and pathogenicity in non-typhoidal *Salmonella* that needs to be empirically evaluated.

Keywords: non-typhoidal *Salmonella*; oxidative stress response; next generation sequencing; biofilm formation; salmonellae-essential virulence genes

1. Introduction

Non-typhoidal *Salmonella* is a leading cause of foodborne gastroenteritis on the global scale. On the global scale, this foodborne pathogen is responsible for 80 million cases of gastroenteritis annually [1]. The situation in the USA is very similar to the global epidemiological picture of salmonellosis caused by non-typhoidal *Salmonella*. It has been estimated that from 2000 to 2008, non-typhoidal *Salmonella* serovars accounted for 1.2 million laboratory-confirmed illnesses, 19,000 hospitalizations, and 380 deaths each year in the USA [2]. The high incidence rate is made more significant by the fact that there have been no signs of decline in the incidence of salmonellosis over the past 15 years in the US. Intriguingly, infections caused by other five major food-borne pathogens (i.e., *Escherichia coli* O157:H7, *Campylobacter* spp., *Listeria monocytogenes*, *Yersinia enterocolitica*, and *Shigella* spp.) declined substantially over the same time [2].

Infections of humans with non-typhoidal *Salmonella* serovars occur mostly via the human food chain [3,4]. The important human food chain transmission route of non-typhoidal *Salmonella* is associated with ready-to-eat products such as vegetables [5,6], leafy green horticulture products [7,8], and fruits [9,10]. Traditionally, aqueous chlorine and ozone solutions are widely used in the food industry for sanitization of food-processing surfaces as well as vegetables and fruits prior to their distribution to the market [11,12]. Chlorine and ozone, potent oxidizing agents, impose an oxidative stress on cells, damaging nucleic acids, both DNA and RNA, proteins, lipids, and further causing membrane disorganization and genome breaks which result in cellular death. To survive oxidative stress assault in the food matrix and food processing facilities and successfully invade the host and survive intracellularly, these pathogens must acquire a robust oxidative stress protective mechanism.

A number of studies investigated the roles of transcription factors [13], RNA repair systems [14], sigma factors [15], and novel stress response proteins [16,17] in the oxidative stress response of non-typhoidal *Salmonella*. Although these studies gave us extremely valuable information on the functions of specific oxidative response mechanisms employed by these pathogens, they could not provide insights into the global impact of oxidative stress on the non-typhoidal *Salmonella* physiology. Recently, Fu et al. [18] by employing global comparative proteomics, identified 116 proteins in the proteome of *Salmonella* Typhimurium, treated with H_2O_2 that were significantly altered compared to the control. Among the altered proteome, iron acquisition systems were most significantly induced. Also, several phage-coding proteins with DNA repair capabilities were induced, whereas the apparatus of the *Salmonella* type III secretion system was repressed.

To gain insight into the expression dynamics of genes associated with distinct biological functions during an oxidative stress, expression of ten genes, including *iroN*, *sitA*, *rpoS*, *rpoH*, *ycfR*, *dps*, *nrdM*, *pocR*, and *ompF*, were tested after 30 min of 1 mM, 2 mM, and 4 mM H_2O_2 treatments. The 30 min of H_2O_2 treatment was selected as *Salmonella enterica* subspecies *enterica* serovar Enteritidis, hereinafter *Salmonella* Enteritidis cultures were already adapted at that point to different H_2O_2 treatments, showing only a slight decline in the viability. Finally, to determine the global impact of oxidative stress on the non-typhoidal *Salmonella* physiology, we carried out comparative transcriptome analyses using the next generation sequencing approach.

2. Materials and Methods

2.1. Bacterial Culture

Salmonella Enteritidis ATCC 13076 (American Type Culture Collection, Manassas, VA, USA) was used as the model organism. For each experiment, fresh culture was prepared by streaking frozen culture onto tryptic soy agar (EMD Chemicals Inc., Darmstadt, Germany), followed by incubation at 37 °C for 24 h. Three well-isolated colonies were used for subsequent inoculations.

2.2. Oxidative Stress Killing Assay

This assay was carried out using overnight cultures of the wild type *S*. Enteritidis strain grown in LB at 37 °C with continuous shaking at 180 rpm. These overnight cultures were diluted 1/100 in LB followed by growth to 0.4 using an optical density at 600 nm. The wild type cultures were exposed at their mid-exponential growth phase (0.4) to three incrementally increasing concentrations of H_2O_2, including: 1 mM, 2 mM, and 4 mM. Treatment was carried out for 90 min under the same incubation conditions. Wild type culture with no oxidative treatment was included as a control. Colony forming units (CFU) counts were carried out by 10-fold dilutions using sterile saline at time zero (before H_2O_2 exposure) and then at every 15 min of treatment. Aliquots of 0.1 mL were plated in triplicate followed by incubation at 37 °C for 24 h.

2.3. Incremental Oxidative Stress Gene Expression Assay

Cultures of *S.* Enteritidis were prepared as described above and after 30 min of oxidative treatment samples were taken and processed. Total RNA was isolated as described below followed by cDNA synthesis using Superscript™ III reverse transcriptase (RT) (Invitrogen by Life Technologies, Carlsbad, CA, USA). Quantitative PCR was carried out on a MiniOpticon™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with iQ™ SYBR Green Supermix kit (Bio-Rad Laboratories) as described previously [19]. Genes *rtcR* and *yjjA*, encoding for transcriptional regulatory protein RtcR and DUF2501 domain-containing protein, were selected as the internal reference genes to normalize the expression of the tested genes. Primers were designed to target expression of the following genes: *rpoS, rpoH, iroN, sitA, dps, nrdM, trxC, ycfR, ompF*, and *pocR* (Table 1). These ten genes were selected based on their distinct gene ontologies (GO) associated with the oxidative stress response. Selected the GO include, iron homeostasis, alternative sigma factors, multiple stress response, DNA protection, antioxidants, DNA replication, uptake of small solutes and anabolic processes. Melting curves were analyzed to check the product specificity. The level of gene expression for each target gene was compared with the internal reference control gene and the fold changes in expression were calculated by the comparative *CT* method [20].

Table 1. Primers used in this study for the oxidative stress response gene expression assay and validation of RNA-seq data.

Gene Names	Primers for the Oxidative Stress Response Gene Expression Assay	
	Forward Sequence (5′–3′)	**Reverse Sequence (5′–3′)**
rpoS	CGA AAA AGC GTT GCT GGA CA	ATC GTC ATC TTG CGT GGT GT
rpoH	CCT TCG CCG TAC ACT GGA TT	GCT TTC GTG GTT GCG ACT TT
iroN	TCA TGG AAA ATG ACC CCG CA	ACC GCG TTC GAA GTA CTG TT
sitA	CGC CAA AAC AGG TGC GTA AA	ATC GGA AAC CGT ACT CTC GC
dps	AAA AGC GAC GGT TGA GTT GC	TAC GGA AGC CAT CCA GCA TC
nrdH	TGG TGA ACG TCG ATC TGG TG	CGG GTG CAG ACG GTT AAT CA
trxC	AGC GGT AAA GTC CGT TTC GT	GAA AGG CGC TTT AGG CAC TG
ycfR	CCG TTG AAG TTC AGG CAA CG	CCC ATC TCC TGC GCT TTT TG
ompF	CAG CGT ACA GCA ACA GCA AG	TCA GCA TAT ACG GCA GCC AG
pocR	GTG GGT AAA CCG CCA GAG AA	AGG TCT GGC GGA AGA CTT TG
Primers for Validation of the RNA-Seq Data		
dinP	TTA CGG GCG GTG GTA ATT AAG	AAG GCT GCG TAA ATC GGT AG
osmY	AAC TCT GCT GGC CGT AAT G	AGG GTG ACG ACT TTC TGA TTG
dnaJ	TGG ATC TCA CCC TGG AAG AA	GCC CAT GAC CGT GAC ATT TA
mdlA	CTC CAG TTG CTG ATA GCG ATA C	GCC AGC GTA CAT GAG GAT ATT
eutC	CTC AAA GAA GTG CCG GAA GA	GGC AGG ATC TCT TCA TAG TTG G
spaR	GAA AGA GAG TCG CGG TAC ATT	CCT TCA TTA CCG CCG CTA TTA
nrdD	CTA TCT GCC CGT TGC CTA AAT	GCT GGA AGA TGT CTG CGG ATT A C
malK	CGA GCG TAC AGC TAC GAA AT	AGA GCG CAT AAG ACT GGA ATA C
pagO	CCC GAG ATA CAG GGT AGC TAA TA	CAG ATC GCG GGC TTA ACT ATC

2.4. Preparation of Samples for Transcriptomics

Untreated and 30-min H_2O_2 (3 mM) treated cultures (0.4 at OD_{600}) of *S.* Typhimurium were centrifuged at 4000 rpm for 5 min. Supernatants were discard and the cell pellets were washed two times followed by RNA extraction using the RNeasy Mini kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions.

2.5. Global Transcriptomic Analysis

Sample quality preparation was done using capillary electrophoresis (Agilent Bio Analyzer 2100, Santa Clara, CA, USA) as previously described [17]. Only samples that had RNA integrity number 8

or greater were considered for further analysis. Illumina sequencing libraries were prepared using Illumina's TruSeq Stranded Total RNA Library Prep Human/Mouse/Rat Sample Preparation Kit (Cat. # 20020597) at the University of Minnesota Genomics Center (Saint Paul, MN, USA). For rRNA depletion, 500 ng of total amount of RNA was used in combination with Ribozero capture probes. After this step, the mRNA was fragmented followed by reverse transcription into complementary DNA (cDNA). The resulting cDNA fragments were coded with indexed adaptors followed by amplification using 15 PCR cycles. Again capillary electrophoresis was used to validate library size distribution, while the library quantification was carried out using fluorimetry. Libraries normalization was carried out followed by their hybridization to a single read flow cell. Once hybridization completed, the flow cell was loaded on the HiSeq 2500 and sequenced using Illumina's SBS chemistry. The primary analysis and index de-multiplexing were performed as previously described [17]. Read mapping was carried out using publicly available genome of *S.* Enteritidis P125109 strain. Quantification of gene expression was completed using Feature Counts. Significantly differentially expressed genes were identified using the edgeR feature in CLCGWB (Qiagen, Valencia, CA, USA) based on a minimum 2 times absolute fold change difference and $p < 0.05$ false discovery rate (FDR).

2.6. Validation of RNA-Seq Data by Real-Time PCR

Cultures of *S.* Enteritidis were prepared as described above and after 30 min of oxidative treatment samples were taken and processed. Total RNA was isolated as described above. Quantitative PCR assay was carried out as described above. Genes *rtcR* and *yjjA* were selected as the internal reference genes to normalize the expression of the tested genes. Primers were designed to target expression of the following genes: *dinP*, *dnaJ*, *eutC*, *mdlA*, *osmY*, *pagO*, *spaR*, *malK*, and *nrdD* (Table 1).

2.7. Experimental Replication and Gene Ontology Analysis

The data of all experiments represent the average of three biological replications. The cell viability during the oxidative treatments were analyzed by CoStat version 6.4 software (Co-Hort Software, Monterey, CA, USA) using the homogeneity of linear regression slopes approach. The gene ontology (GO) analysis was carried out using the Database for Annotation, Visualization and Integrated Discovery (DAVID) database [21] as well as the National Center for Biotechnology Information (NCBI).

2.8. RN-Seq Accession Numbers

RNA sequencing data for all three biological replications and two different treatments were deposited in the NCBI under accession number GSE 155479.

3. Results

3.1. Effect ofIincreasing H_2O_2 Concentrations on the Viability of S. Enteritidis

To evaluate the ability of *S.* Enteritidis to survive exposure to oxidative stress, we performed oxidative stress killing assays with increasing concentrations of H_2O_2 over a short time. The concentration of 1 mM of H_2O_2 caused a death rate of 0.37 $\log_{10}$ CFU for the first 15 min of exposure (Figure 1 and Figure S1). After this initial lethal period, the culture of *S.* Enteritidis exposed to the same concentration of H_2O_2 showed a slight decline in the number of viable cells, resulting in only 0.1 $\log_{10}$ CFU of reduction during the entire experiment (Figure 1). The exposure of *S.* Enteritidis to 1 mM of H_2O_2 resulted in two distinct survival phases, an initial and brief lethal phase followed by a long bacteriostatic phase (Figure 1 and Figure S1). Similarly to 1 mM, the concentration of 2 mM of H_2O_2 caused an initial lethal phase resulting in 0.53 $\log_{10}$ CFU decline (Figure 1). However, the survival pattern associated with the 2 mM H_2O_2 treatment was bactericidal not bacteriostatic (Figure 1). After an initial lethal period for the first 15 min of H_2O_2 exposure, the 2 mM H_2O_2 treatment continued to cause cell death, albeit at a slower rate compared to that of the initial 15-min period. This bactericidal phase was characterized by repetitive fluctuations of fast die-off periods (e.g., 0.23, 0.24 and 0.22 $\log_{10}$

CFU declines at the 30-min, 60-min, and 90-min measurements, respectively) and slight growth periods (e.g., 0.06 $\log_{10}$ CFU increase at the 45-min measurement and 0.03 $\log_{10}$ CFU increase at the 75-min measurement) (Figure 1). The exposure of *S.* Enteritidis to the greatest concentration of H_2O_2, 4 mM, caused again an initial lethal phase, characterized by a fast die-off rate, 0.72 $\log_{10}$ CFU decline over the first 15 min of treatment (Figure 1). Interestingly, this initial fast die-off period was followed by moderate die-off periods (e.g., 0.18 and 0.17 $\log_{10}$ CFU decline) at the next two measurements (Figure 1). After this moderate die-off period, the culture of *S.* Enteritidis entered progressively fast mortality rates resulting in a 0.38 $\log_{10}$ CFU decline at the 60-min measurement and 1.19 $\log_{10}$ CFU decline at the 75-min measurement (Figure 1). At the last, 90-min, measurement, the viable cell count could not be detected at 10^3 CFU per 1 mL, indicating a continuation of this progressive and fast mortality rate. The oxidative treatments with 1 mM, 2 mM, and 4 mM of H_2O_2 caused significantly higher ($p < 0.01$) mortality rates during the exponential growth phase of *S.* Enteritidis compared with no treatment of this pathogen under the same growth conditions. The average mortality rates of *S.* Enteritidis for every 15 min of H_2O_2 treatment were 0.08 $\log_{10}$ CFU decline for 1 mM, followed by 0.188 $\log_{10}$ CFU decline for 2 mM treatment and 0.468 $\log_{10}$ CFU decline for 4 mM. Although all three H_2O_2 treatments caused significantly higher mortality rates compared to that of no treatment, the survival patterns of H_2O_2 treated *S.* Enteritidis were different (Figure 1).
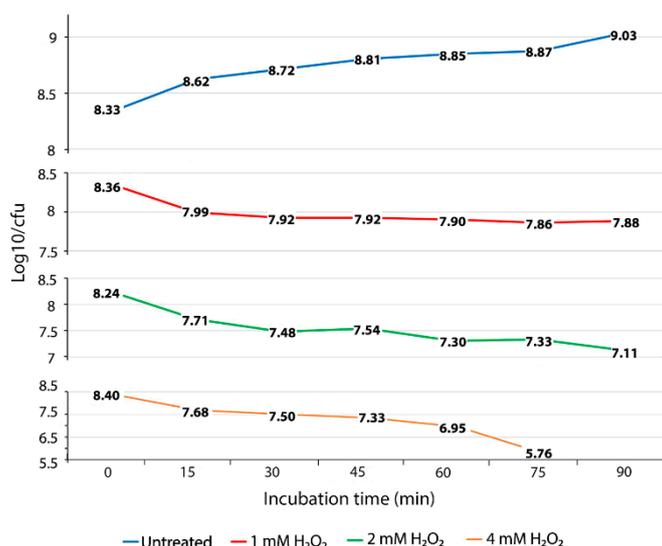
Figure 1. Oxidative stress killing assay. Mortality of *S.* Enteritidis, expressed by reduced $\log_{10}$/CFU for every 15 min of the H_2O_2 treatment. The data correspond to the mean value of three biological replications. Each measurement value was composed of nine replications (each biological replication was made up of three technical replications).

3.2. Expression Levels of the Genes Associated with Oxidative Stress and Anabolic Processes during Incremental Increase of H_2O_2 Concentration

To determine the relationship between a death rate of *S.* Enteritidis and expression of genes that are associated with the most important oxidative stress adaptive processes, we carried out a qRT-PCR assay. The gene expression assay was carried out after 30 min of H_2O_2 treatment when *Salmonella* cells have already been adapted to the oxidative stressor (Figure 1). The targeted genes were selected from the following stress response and anabolism associated processes, iron homeostasis (*iroN*, *sitA*), alternative sigma factors (*rpoS*, *rpoH*), multiple stress responses (*ycfR*), DNA protection (*dps*), antioxidants (*trxC*), DNA replication (*nrdM*), uptake of small solutes (*ompF*) and anabolic process (*pocR*).

No significant changes in expression of *rpoS* were observed during 1 mM and 2 mM H_2O_2 treatment, whereas 4 mM H_2O_2 treatment caused a significant (2.5-fold) downregulation of the *rpoS* gene (Figure 2). Similarly to the *rpoS* gene, the oxidative treatments of *S.* Enteritidis caused minor

alterations in the expression of the gene that encodes another stress response sigma factor, RpoH (Figure 2). The 1 mM and 4 mM treatments caused 2-fold and 2.9-fold upregulation of the *rpoH* gene, while 2 mM treatment caused no significant (1.4-fold) upregulation of the same gene.
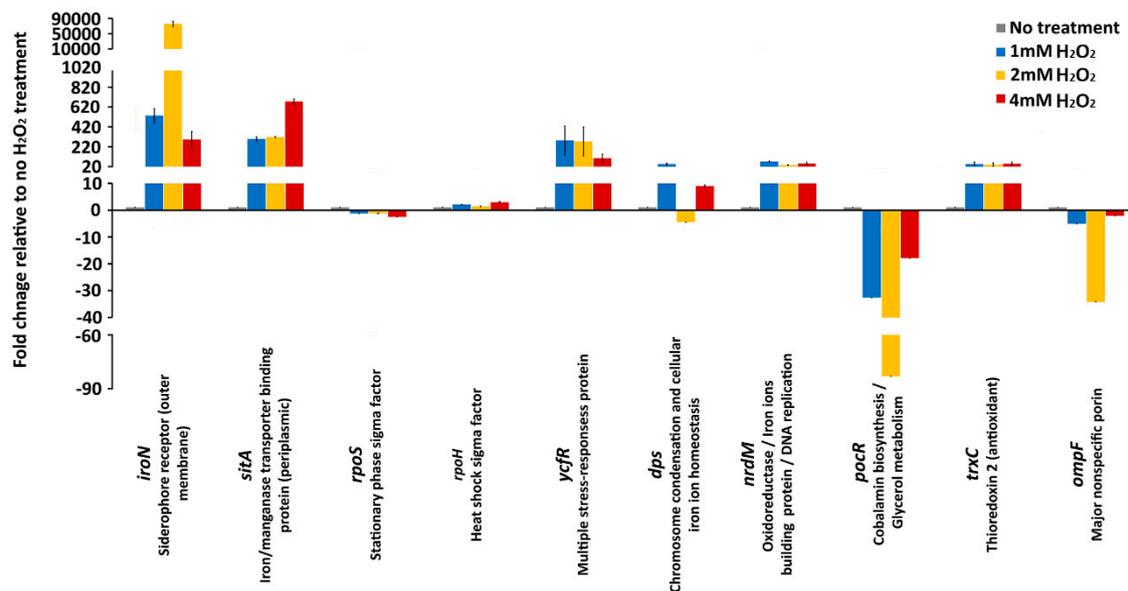
Figure 2. Gene expression. mRNA expression levels of *iroN*, *sitA* (iron receptor), *rpoS*, *rpoH* (alternative sigma factors), *ycfR*, *dps* (stress response), *nrdM*, *trxC* (antioxidant), *ompF* (nonselective uptake), and *pocR* (anabolism) during no H_2O_2 treatment, 1 mM, 2 mM, and 4 mM H_2O_2 treatments. Values on the y-axis are relative expression levels (fold change) normalized against the level in the no H_2O_2 treatment group. The data correspond to the mean values of three biological replications. Error bars correspond to the standard deviation.

In sharp contrast to the genes that encode stress response sigma factors, the genes associated with iron homeostasis, in particular genes that encode the iron receptors, IroN (out membrane receptor) and SitA (periplasmic receptor) showed the greatest gene expression alterations (Figure 2). Most notably, the expression of *iroN* exhibited a profound upregulation with the 1 mM H_2O_2 treatment (532-fold) and with 2 mM H_2O_2 treatment, the upregulation of this gene was extraordinarily high (75664-fold) compared to that of the H_2O_2 untreated control. Interestingly, with the greatest concentration of oxidative stressor, 4 mM, the upregulation of the *iroN* gene was lower (292-fold) than that with 1 mM (532 fold) and 2 mM (75664 fold) treatment (Figure 2). Similarly, other genes involved in the iron homeostasis, *sitA*, showed large upregulation upon the oxidative stress treatment with 1 mM (298-fold), 2mM (316-fold) and 4 mM (675-fold) H_2O_2 treatment. Although both genes encode for iron receptors, their expression (stress response) patterns were different. The upregulation of the *sitA* gene was proportional to incremental increases of H_2O_2 concentration, whereas the upregulation of the *iroN* gene did not follow the level of H_2O_2 concentration (Figure 2).

The *ycfR* gene, which encodes a multiple stress response protein YcfR, was upregulated throughout the H_2O_2 treatments. The upregulation of the *ycfR* gene ranged from 283-fold and 273-fold to 103-fold in the *Salmonella* cultures exposed to 1 mM, 2 mM, and 4 mM H_2O_2, respectively (Figure 2). Similarly to the *iroN* gene, the greatest concentration of H_2O_2, 4 mM, caused the lowest upregulation of the *ycfR* gene, further indicating to some extent the similar patterns of the responses of these two oxidative stress response associated genes. However, the *ycfR* gene did not show an extremely high upregulation during the 2 mM H_2O_2 treatment, as the *iroN* gene showed (Figure 2). Another stress response gene, *dps*, had an unusual expression profile. During treatment with 1 mM H_2O_2 this gene was upregulated 44-fold, then during the 2 mM treatment the *dps* gene was downregulated 4-fold, and again upregulated 8.9-fold with 4 mM H_2O_2 treatment (Figure 2). This unusual expression profile of

the *dps* gene, which encodes a protein that protects DNA from reactive oxygen species (ROS), indicates a complex interaction of the oxidative stress response associated genes.

The genes associated with DNA replication and oxidoreductase processes, *nrdM* and *trxC*, had similar genes expressions profiles (Figure 2). Both genes exhibited 35- to 70-fold upregulation during the oxidative treatments (Figure 2). The *ompF* gene, which encodes for a major nonspecific OmpF porin, and the *pocR* gene, which is associated with anabolic processes, exhibited downregulation during the oxidative treatments (Figure 2). Interestingly, the downregulation gene expression patterns for both the *ompF* and *pocR* genes were the same. The greatest gene alterations (e.g., downregulation) were observed during 2 mM H_2O_2 treatment, whereas the 4 mM H_2O_2 treatment caused the lowest level of the gene expression alteration for both genes (Figure 2).

3.3. Global Transcriptome Response of S. Enteritidis to Oxidative Stress.

After the determination of the stress response patterns of a limited number of genes during treatment with different concentrations of H_2O_2, we carried out the global transcriptome analyses using a single concentration of H_2O_2. In total, 2051 genes were significantly differently expressed (SDE) in *S*. Enteritidis during the 3 mM H_2O_2 treatment compared to the reference transcriptome of the same *S*. Enteritidis strain with no H_2O_2 treatment. Of the 2051 SDE genes, 1111 were induced and 941 genes were repressed, showing at least a 2-fold alteration in gene expression and high reproducibility (false discovery rate < 0.5) across all three biological replications (Tables S1 and S2).

3.3.1. Induction of Transcriptome—Molecular Response of *S*. Enteritidis to Oxidative Stress

The most altered change in biological processes of oxidatively stressed *S*. Enteritidis cells was the induction of genes encoding Fe-S cluster biogenesis (Tables S1 and S2). Operons encoding sulfur utilization factor (SUF), *sufABCSD* and *ynhAG*, were upregulated from 173- to 903-fold and from 23- to 114-fold, respectively (Figure 3). The SUF operons were profoundly more induced compared to ones encoding iron-sulfur cluster (ISC), *nifJUS*, *yfhFP* and *hscBA* (Figure 3). Another group of highly induced genes were associated with the iron ion homeostasis. The operon *sitABCD*, iron/manganese ABC transporter, showed the greatest induction among operons/genes associated with this biological function (Figure 3). Besides this Fe/Mn transporter, a group of operons/genes encoding a wide range of iron transporters showed the greatest inductions, including *iroN*, ferric salmochelin siderophore receptor; *fepBD*, ferric enterobactin ABC transporter; *fhuABCD*, ferrichrome outer membrane transporter; *ydiE*, hemin transporter; *exbBD*, iron siderophore bacteriocin transporter; *fhuE*, ferric coprogen and ferric-rhodotorulic acid receptor as well as *fhuF*, receptor for ferrioxamine B (Figure 3). Also, several operons and a gene responsible for the biosynthesis of salmochelin (*iroBCE*), enterobactin (*ybdABZ*, *entABCDEF*) and hem (*hemH*) were greatly induced during oxidative stress (Figure 3).

The significant induction showed a group of operons/genes encoding proteins that govern the cell-redox homeostasis. Among the cell-redox encoding genes, the *nrdH* gene which encodes a classical antioxidant, glutaredoxin-like protein NrdH, showed the greatest induction, resulting in 144.3-fold upregulation compared to the reference strain (Figure 4). The *trxC* gene that encodes an effective cytoplasmic disulfide-reducing protein, thioredoxin 2, showed 76-fold induction (Figure 4). The *grxAB* operon, encoding the antioxidants glutaredoxin 1 and 2, showed a moderate induction compared to that of the *nrdH* gene. A major part of the cell-redox regulon was associated with the genes encoding proteins associated with the electron transport chain (Figure 4). The *narIJHKG* operon, involved in nitrate assimilation and anaerobic electron transport, exhibited the greatest induction among this group of genes, followed by *fdoHGI*, dehydrogenases that transfer electrons from the periplasmic to the cytoplasmic heme; *sdhABCD*, cytochrome b556; *cyoABCDE*, cytochrome bo3 oxidase complex and *sthA*, which converts NADPH to NADH (Figure 4).

Induced

2-4	5-7	8-10	11-15	16-20	21-25	26-30	31-40	41-50	51-60	61-100	>100

Gene	Protein function/name	Fold change	Biological function
sufA	Fe-S cluster assembly scaffold SufA		
sufB	Fe-S cluster assembly protein SufB		
sufC	Fe-S cluster assembly ATPase SufC		Iron-sulfur cluster assembly (SUF system)
sufS	Bifunctional cysteine desulfurase/selenocystein lyase SufS		
sufD	Fe-S cluster assembly protein SufD		
ynhA	Cysteine desufuration protein SufE		
ynhG	Putative L,D-transpeptidase YnhG		
nifJ	Pyruvate-flavodoxin oxidoreductase IscJ		
nifU	Fe-S cluster assembly scaffold IscU		
nifS	Cysteine desulfurase IscS		Iron-sulfur cluster assembly (ISC system)
yfhF	Iron-sulfur cluster assembly protein IscA		
yfhP	Fe-S cluster assembly transcriptional regulator IscR		
hscA	Fe-S protein assembly chaperone HscA		
hscB	Fe-S protein assembly co-chaperone HscB		
sitA	Iron/manganese transporter, periplasmic-binding protein SitA		
sitB	Iron/manganese transporter, inner membrane protein SitB		
sitC	Iron/manganese transporter, inner membrane protein SitC		
sitD	Iron/manganese transporter, inner membrane permease SitD		
iroN	Salmochelin siderophore system receptor protein IroN		
fepB	Fe^{2+}-enterobactin ABC transporter binding protein FepB		
fepD	Ferric enterobactin transport protein FepD		
fhuA	Ferrichrome-iron receptor FhuA		
fhuB	Ferrichrome-iron ABC transporter permease FhuB		
fhuC	Ferrichrome-iron transport ATP-binding protein FhuC		
fhuD	Ferrichrome-iron binding periplasmic protein FhuD		
fhuE	Ferric-rhodotorulic acid outer membrane transporter FhuE		
fhuF	Siderophore-iron reductase FhuF		
ydiE	Hemin uptake protein HemP		
exbB	Biopolymer transport protein ExdB		Iron homeostasis
exbD	Biopolymer transport protein ExdD		
iroB	Salmochelin siderophore glycosyltransferase protein IroB		
iroC	Multidrug ABC transporter ATP-binding protein IroC		
iroE	Salmochelin siderophore system protein IroE		
ybdA	Enterobactin exporter EntS		
ybdB	Thioesterase YbdB		
ybdZ	Enterobactin biosynthesis protein YbdZ		
entA	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase EntA		
entB	Isochorismatase EntB		
entC	Isochorismate synthase EntC		
entD	Enterobactin synthase component D protein EntD		
entE	Enterobactin synthase component E protein EntE		
entF	Enterobactin synthase component F protein EntF		
hemH	Ferrochelatase HemH		

Figure 3. Differential expression of genes involved in iron-cluster assembly and iron ion homeostasis. Transcriptional profiling of the wild type *S.* Enteritidis ATCC 13076. Isolation of RNA was carried out during the exponential growth phase. The transcriptomic profiles were determined using a HiSeq 4000 PE100 Illumina platform. The mean fold-increase of three biological replications in each gene expression after exposure to 3 mM of H_2O_2 compared with controls (no H_2O_2 treatment and the same growth phase) is indicated by the color scale bar. The biological functions of the induced gene were determined using the Database for Annotation, Visualization and Integrated Discovery (DAVID) as well as the National Center for Biotechnology Information (NCBI). Both sulfur utilization factor (SUF) and iron-sulfur cluster (ISC) belong to the same biological function "Iron-sulfur cluster assembly".
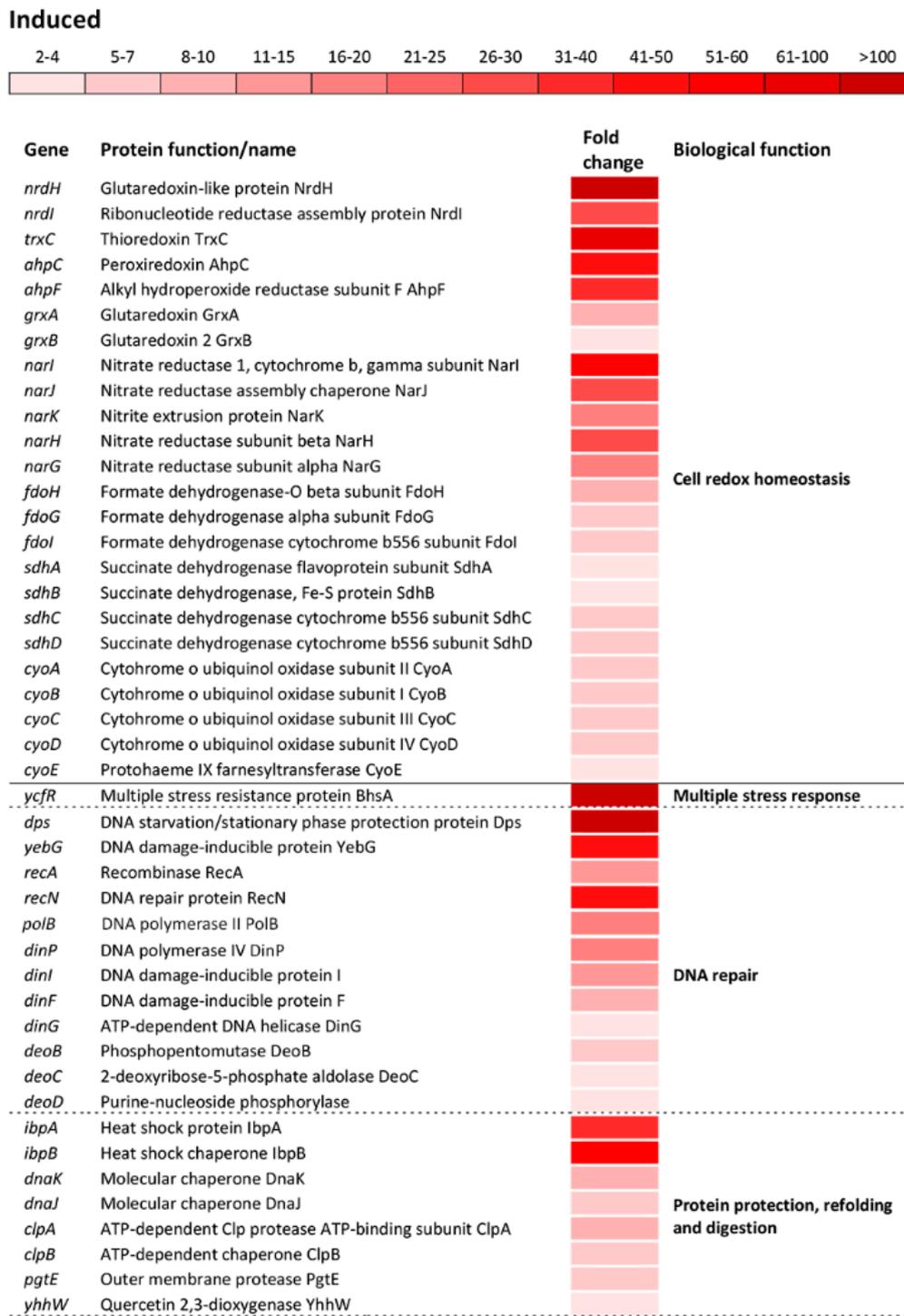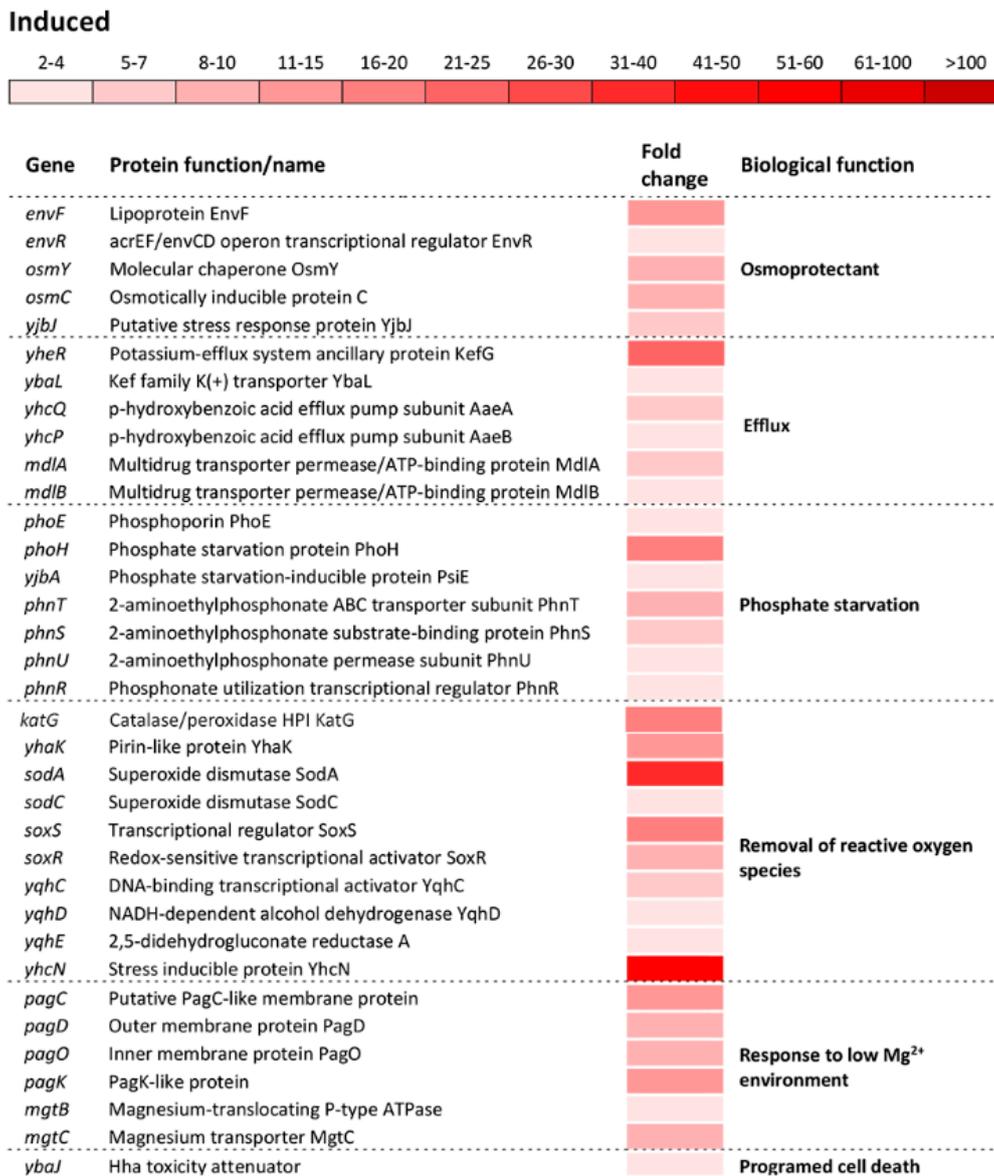
Induced

2-4	5-7	8-10	11-15	16-20	21-25	26-30	31-40	41-50	51-60	61-100	>100

Gene	Protein function/name	Fold change	Biological function
nrdH	Glutaredoxin-like protein NrdH		
nrdI	Ribonucleotide reductase assembly protein NrdI		
trxC	Thioredoxin TrxC		
ahpC	Peroxiredoxin AhpC		
ahpF	Alkyl hydroperoxide reductase subunit F AhpF		
grxA	Glutaredoxin GrxA		
grxB	Glutaredoxin 2 GrxB		
narI	Nitrate reductase 1, cytochrome b, gamma subunit NarI		
narJ	Nitrate reductase assembly chaperone NarJ		
narK	Nitrite extrusion protein NarK		
narH	Nitrate reductase subunit beta NarH		
narG	Nitrate reductase subunit alpha NarG		
fdoH	Formate dehydrogenase-O beta subunit FdoH		Cell redox homeostasis
fdoG	Formate dehydrogenase alpha subunit FdoG		
fdoI	Formate dehydrogenase cytochrome b556 subunit FdoI		
sdhA	Succinate dehydrogenase flavoprotein subunit SdhA		
sdhB	Succinate dehydrogenase, Fe-S protein SdhB		
sdhC	Succinate dehydrogenase cytochrome b556 subunit SdhC		
sdhD	Succinate dehydrogenase cytochrome b556 subunit SdhD		
cyoA	Cytochrome o ubiquinol oxidase subunit II CyoA		
cyoB	Cytochrome o ubiquinol oxidase subunit I CyoB		
cyoC	Cytochrome o ubiquinol oxidase subunit III CyoC		
cyoD	Cytochrome o ubiquinol oxidase subunit IV CyoD		
cyoE	Protohaeme IX farnesyltransferase CyoE		
ycfR	Multiple stress resistance protein BhsA		**Multiple stress response**
dps	DNA starvation/stationary phase protection protein Dps		
yebG	DNA damage-inducible protein YebG		
recA	Recombinase RecA		
recN	DNA repair protein RecN		
polB	DNA polymerase II PolB		
dinP	DNA polymerase IV DinP		
dinI	DNA damage-inducible protein I		DNA repair
dinF	DNA damage-inducible protein F		
dinG	ATP-dependent DNA helicase DinG		
deoB	Phosphopentomutase DeoB		
deoC	2-deoxyribose-5-phosphate aldolase DeoC		
deoD	Purine-nucleoside phosphorylase		
ibpA	Heat shock protein IbpA		
ibpB	Heat shock chaperone IbpB		
dnaK	Molecular chaperone DnaK		
dnaJ	Molecular chaperone DnaJ		Protein protection, refolding and digestion
clpA	ATP-dependent Clp protease ATP-binding subunit ClpA		
clpB	ATP-dependent chaperone ClpB		
pgtE	Outer membrane protease PgtE		
yhhW	Quercetin 2,3-dioxygenase YhhW		

Figure 4. *Cont.*

Induced

2–4	5–7	8–10	11–15	16–20	21–25	26–30	31–40	41–50	51–60	61–100	>100

Gene	Protein function/name	Fold change	Biological function
envF	Lipoprotein EnvF		
envR	acrEF/envCD operon transcriptional regulator EnvR		
osmY	Molecular chaperone OsmY		Osmoprotectant
osmC	Osmotically inducible protein C		
yjbJ	Putative stress response protein YjbJ		
yheR	Potassium-efflux system ancillary protein KefG		
ybaL	Kef family K(+) transporter YbaL		
yhcQ	p-hydroxybenzoic acid efflux pump subunit AaeA		Efflux
yhcP	p-hydroxybenzoic acid efflux pump subunit AaeB		
mdlA	Multidrug transporter permease/ATP-binding protein MdlA		
mdlB	Multidrug transporter permease/ATP-binding protein MdlB		
phoE	Phosphoporin PhoE		
phoH	Phosphate starvation protein PhoH		
yjbA	Phosphate starvation-inducible protein PsiE		
phnT	2-aminoethylphosphonate ABC transporter subunit PhnT		Phosphate starvation
phnS	2-aminoethylphosphonate substrate-binding protein PhnS		
phnU	2-aminoethylphosphonate permease subunit PhnU		
phnR	Phosphonate utilization transcriptional regulator PhnR		
katG	Catalase/peroxidase HPI KatG		
yhaK	Pirin-like protein YhaK		
sodA	Superoxide dismutase SodA		
sodC	Superoxide dismutase SodC		
soxS	Transcriptional regulator SoxS		Removal of reactive oxygen species
soxR	Redox-sensitive transcriptional activator SoxR		
yqhC	DNA-binding transcriptional activator YqhC		
yqhD	NADH-dependent alcohol dehydrogenase YqhD		
yqhE	2,5-didehydrogluconate reductase A		
yhcN	Stress inducible protein YhcN		
pagC	Putative PagC-like membrane protein		
pagD	Outer membrane protein PagD		
pagO	Inner membrane protein PagO		Response to low Mg^{2+} environment
pagK	PagK-like protein		
mgtB	Magnesium-translocating P-type ATPase		
mgtC	Magnesium transporter MgtC		
ybaJ	Hha toxicity attenuator		Programed cell death

Figure 4. Differential expression of genes involved in cell redox homeostasis and various stress responses. The mean fold-increase in each gene expression after exposure to 3 mM of H_2O_2 compared with controls is indicated by the color scale bar. Note that a variety of biological functions including multiple stress response, DNA repair, protein protection, refolding and digestion, osmoprotectants, efflux, phosphate starvation, removal of reactive oxygen species, response to low Mg^{2+} environment and programed cell death belong to a more general biological function called "Stress response".

The most numerous group of altered operons/genes in the transcriptome of oxidatively stressed *S.* Enteritidis cells was associated with various stress response processes as a distinct biological function indicated by the GO analysis. The greatest alteration in the entire transcriptome of *S.* Enteritidis under H_2O_2 stress was related to the *ycfR* gene. This gene, encoding the multiple stress resistance outer membrane protein YcfR, was induced 913-fold compared to that of the reference strain (Figure 4). The great induction exhibited the *dps* gene, which encodes the DNA-binding protein Dps that protects DNA. Besides *dps*, another group of genes encoding DNA repair proteins was significantly induced, including the DNA damage inducible *yebG* gene and the *recAN*, *polB*, *dinPIG*, and *deoBCD* genes (Figure 4). Similarly to genes encoding DNA repair proteins, a group of genes encoding molecular chaperones and proteases was significantly induced. Among this group of genes, the *ibpBA* gene,

encoding small heat shock proteins, showed the greatest induction, followed by molecular chaperones *dnaKJ, yhhW*, and *clpAB*, and protease *pgtE* (Figure 4).

The oxidative stress response regulon included upregulation of: Osmotically inducible genes *envFR*, *osmYC*, and *yjbJ*; efflux encoding genes *yheR*, *yabL*, *yhcQP, dinF*, and *mdlAB*; phosphate starvation inducible genes *phoHE, yjbA*, and *phnTSUR*; sensing and removal of ROS *katG, ahpCF, yhaK, sodAC, soxSR, yqhACDE*, and *yhcN*; adaptation to low Mg^{2+} environment *pagCDOK* and *mgtBC;* and the programmed cell death inducible gene *ybaJ* (Figure 4).

The oxidative stress caused the induction of numerous operons/genes located on *Salmonella* pathogenicity islands (SPIs). The significant induction was observed for the *ssrAB* operon that positively controls the expression of the SPI-2 genes (Figure 5)

Induced

| 2-4 | 5-7 | 8-10 | 11-15 | 16-20 | 21-25 | 26--30 | 31-40 | 41-50 | 51-60 | 61-100 | >100 |

Gene	Protein name	Fold change	Biological function
ssrA	Sensor kinase SsrA		
ssrB	DNA-binding response regulator SsrB		
ssaB	Type III secretion system apparatus protein SsaB		
ssaC	Type III secretion system outer membrane ring protein SsaC		
ssaD	Type III secretion system inner membrane ring protein SsaD		
ssaE	Type III secretion system needle protein co-chaperone SsaE		
ssaH	Type III secretion system apparatus protein SsaH		
ssaI	Type III secretion system inner rod protein SsaI		
ssaJ	Type III secretion inner membrane ring protein SsaJ		
ssaK	Type III secretion system protein SsaK		
ssaL	Type III secretion system apparatus protein SsaL		
ssaM	Type III secretion system protein SsaM		
ssaN	Type III secretion system ATPase SsaN		
ssaQ	Type III secretion system protein SsaQ		
ssaV	Type III secretion system export apparatus protein SsaV		
sifA	Effector protein SifA		
sifB	Effector protein SifB		**Pathogenesis**
sseAb	Pathogenicity island II effector protein SseAb		
sseBb	Pathogenicity island II effector protein SseBb		
sseJ	Pathogenicity island II effector protein SseJ		
sseF	Pathogenicity island II effector protein SseF		
sseI	Secreted effector protein SseI		
sseE	Pathogenicity island II effector protein SseE		
spaQ	Virulence associated secretory protein SsaM		
spaS	Surface presentation of antigens protein SpaS		
spaR	Surface presentation of antigens protein SpaR		
spaO	Surface presentation of antigens protein SpaO		
spaP	Surface presentation of antigens protein SpaP		
iagB	Invasion protein IagB		
sinH	Intimin protein SinH		
sinR	Transcriptional regulator SinR		
sopE2	Invasion-associated secreted effector protein SopE2		
sicP	Secretion chaperone SicP		
yncC	Colanic acid/biofilm transcriptional regulator YncC		
stbA	Fimbrial major subunit StbA		
stbB	Fimbrial chaperone protein StbB		
stbC	Outer membrane fimbrial usher protein StbC		
stbD	Fimbrial protein StbD		
fimD	Outer membrane usher protein FimD		
fimF	Adhesin FimF		
fimH	Fimbrial adhesin FimH		
fimW	Fimbriae W protein		**Cell adhesion**
fimY	Fimbriae Y protein		
sthA	Fimbrial assembly chaperone SthB		
sthB	Outer membrane fimbrial usher protein SthB		
SEN4247	Fimbrial protein SEN4247		
safB	Fimbrial assembly chaperone SafB		

Figure 5. *Cont.*

Figure 5. Differential expression of genes involved in pathogenesis and cell adhesion. The mean fold-increase in each gene expression after exposure to 3 mM of H_2O_2 compared with controls is indicated by the color scale bar.

Indeed, a significant induction of the *ssaMCBDEVJKHILQN* operon encoding the type III secretion system (T3SS) apparatus of the SPI-2 as well as operons *sifAB* and *sseAbJBbFIE* encoding the SPI-2 effector proteins (Figure 5) was observed. Also, *spaQSROP* (translocon), *iagB*, *sinHR*, and *sopE2* (effectors) as well as *sicP* (effector chaperone) located on the SPI-1 were significantly induced (Figure 5). Not only genes encoding the T3SS apparatus and effector proteins responsible for the invasion of host cells and intracellular survival, but also genes encoding various cell adhesions were induced. This group of genes included *yncC*, biofilm transcriptional regulator; *stbABCD*, *fimHYWDF*, *sthAB* and SEN4247, fimbrial operons/genes; *safBCD* and SEN1978, pilin operon/gene as well as *csgABF*, curli operon (Figure 5).

Adaptation to oxidative stress involved induction of operons/genes that encode proteins associated with carbohydrate metabolism *yjfR*, *ydiN*, *nagABCE* (glucosamine catabolic processes), *pduJKLMDQTNOPHUWSECVXG* (carboxysomes formation) and *yiaMOGN* (uptake of gluconate); amino acid metabolism *asnABC*, *yehEWX*, *dadAX*, *yejF*, *lysA*, *yneHIG*, *ybaO*, and *livCKGFMH* (uptake of branched-chain amino acids); nucleoside metabolism *xapAB* (purine salvage pathway); outer membrane modification *pagP*, *nlpD*, *ybaN*, *wcaEFGHID* (colonic acid biosynthesis), *safA*, *ompX* (porin), and *ompA* (porin) and genes encoding proteins of unknown functions (Table S1).

3.3.2. Repression of Transcriptome—Molecular Response of *S.* Enteritidis to Oxidative Stress

There is a distinct segregation based on the biological functions between the induced and repressed transcriptome of oxidatively stressed *S.* Enteritidis cells. While the induced transcriptome showed a great association with the iron-sulfur cluster assembly, iron ion homeostasis, cell redox homeostasis, a variety of stress responses, pathogenesis and sessile lifestyle (cell adhesion), the repressed transcriptome was mainly associated with carbohydrate metabolic processes, ribosomal biogenesis, anaerobic respiration and cell motility (Figure 6).
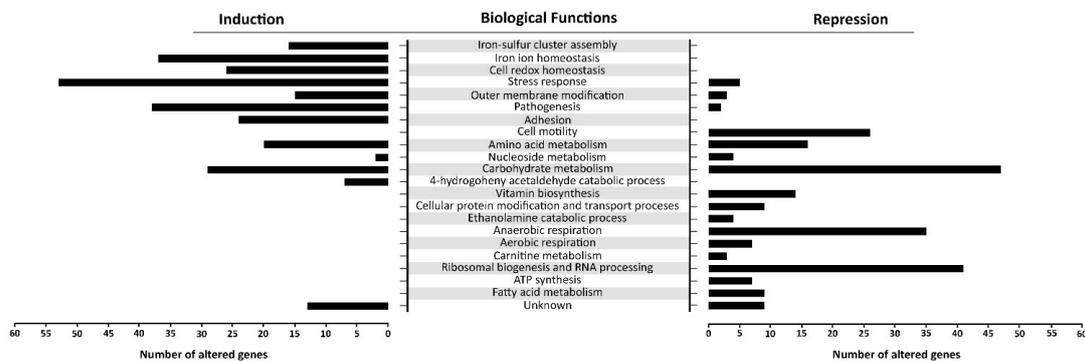
Figure 6. Gene ontology (GO) enrichment analysis. The figure portrays the most altered biological functions of *S.* Enteritidis during the H_2O_2 treatment. In the GO analysis were included operons/genes

that showed the greatest alterations compared to their counterparts in the control sample. Each gene showed false discovery rate (FDR) $p < 0.05$ and high reproducibility across the biological replications.

The *tdcA* gene, encoding a transcriptional activator for the *tdcABCD* operon, showed the greatest repression, resulting in 131-fold downregulation compared to that of the reference strain (Figure 7). Indeed, other genes of the *tdcABCD* operon, which are involved in L-threonine and L-serine catabolic processes, showed significant repression (Figure 7). Also, additional operons/genes associated with amino acid metabolism showed significant repression including *yhdG* (amino acid permease), *gcvTHR* (glycine, serine and threonine metabolism), *pepETQD* (hydrolases of peptide bonds), *selAB* (selenocysteine synthesis) and *sdaBC* (serine uptake and degradation) (Figure 7).

Figure 7. *Cont*.
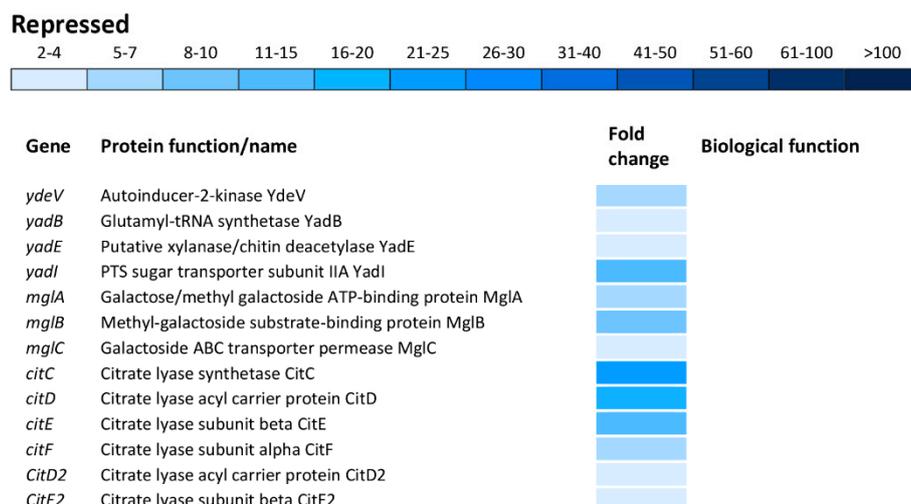
Figure 7. Differential expression of genes involved in amino acid and carbohydrate metabolisms. The mean fold-decrease in each gene expression after exposure to 3 mM of H_2O_2 compared with controls is indicated by the color scale bar.

Carbohydrate metabolism was the biological function most commonly affected within the repressed transcriptome. This group of operons/genes, encoding a wide range of proteins, was involved in catabolic pathways, including *gudDP* (D-glucarate), *garDLKR* (galactarate), *eutSPQT* (ethanolamine), *nanAEKT* (sialic acid), *melABR* (melibiose), *srlAR* (sorbitol), *kdgTK* (D-glucuronate), *idnDT*, and *gntRT* (D-gluconate) (Figure 7). Besides this group of operons/genes associated with catabolism, there was repression of operons/genes encoding sugar uptake and anabolism such as *malFTKMG* (maltose), *ydeWZYV* (carbohydrate permease), *yadBEI* (a major carbohydrate active transport system), *mglABC* (galactose/methyl galactoside) and *citCDEFD2E2* (citric operon involved in anabolic processes of the tricarboxylic acid cycle) (Figure 7).

Other central metabolic processes repressed during oxidative stress of *S. enterica* included anaerobic respiration (*napABCDFGH*, *dmsA2A3A1AB1B*, *glpABCFTXK*, *frdABC*, *nrfABCD*, *hybABCFG*, *deuABC*), ribosomal biogenesis and rRNA processing (*ygjRO*, *rpsUTPLGSOCFHNJQBR*, *rplUKSJNXVBCDAEPLWYM*, *rimJMK*, *rpmAGBHC*), fatty acid metabolic processes (*accBD*, *fadHRL*, *fabIGBAH*), vitamin biosynthesis (*pocR*, *ynfKA*, *cbiACDETF*, *menDAB*, *yfbBSU*), ATP synthesis (*atpBIEHFGA*), cellular protein modification and transport processes (*hypAODBCE* and *oppCBD*), aerobic respiration (*yhbUTCSPQV*), the ethanolamine catabolic process (*eutSPQT*), the carnitine metabolic process (*caiFEA*) and the pyrimidine nucleobase biosynthetic process (*pyrHDLE*) (Figure 6).

3.4. Validation of RNA-seq Data by Real-Time PCR

To validate the RNA-seq data, the expressions of nine randomly chosen genes were tested by quantitative real-time PCR (qRT-PCR) under the same experimental conditions. The qRT-PCR showed the same pattern of expressions, including seven upregulated genes (*dinP*, *dnaJ*, *eutC*, *mdlA*, *osmY*, *pagO*, and *spaR*) and two downregulated genes (*malK* and *nrdD*) (Figure 8). It can be observed that generally, the expression values measured by qRT-PCR were greater compared to those measured by RNA-seq analysis. The difference in expression values between these two techniques is consistent with the fact that qRT-PCR generally gives higher expression values compared to RNA-seq [17,22].
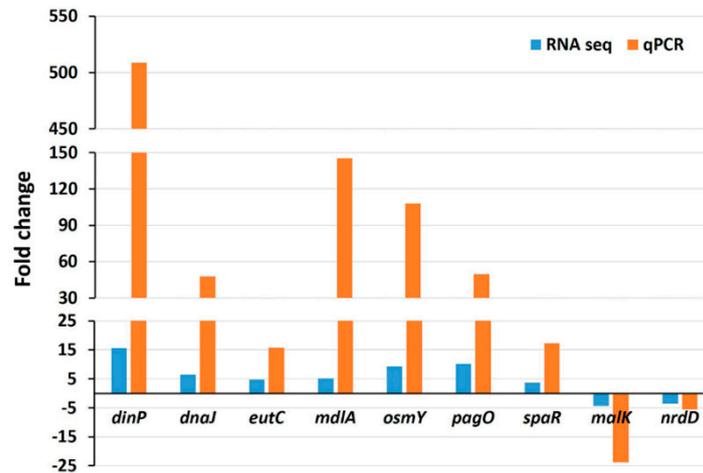
Figure 8. Validation of RNA-seq data by qRT-PCR analysis. Data represent fold changes in the expression of nine randomly selected genes between the wild type strain without treatment and the same strain treated with 3 mM H_2O_2. Genes differently expressed between the wild type strain with no treatment and the wild type with H_2O_2 treatment represent the mean value of three biological replications.

4. Discussion

Salmonella Enteritidis, one of the most important non-typhoidal *Salmonella* serovars from the public health and veterinary medicine perspectives [23–26], has developed a robust anti-oxidative response not only to survive endogenous oxidative stress [27] but also to survive exogenous stress imposed by a host [28] or environment [29]. In other words, for successful host invasion, intracellular proliferation and/or environmental survival, possession of an efficient oxidative stress response is one of the key physiological characteristics of this pathogen. In this study, it was shown that incrementally increased concentrations of oxidant agent caused various patterns of gene expressions. While inductions of genes encoding antioxidant (thioredoxin 2) and DNA replication regulator (NrdM) were constant regardless of H_2O_2 concentrations, induction of a gene encoding iron/manganese transporter, SitA, was proportional with H_2O_2 concentrations. In contrast to the pattern of *sitA* induction, the *ycfR* gene, encoding multiple stress-response protein, exhibited disproportionate induction regarding H_2O_2 concentration. The greatest concentration of H_2O_2 resulted in the lowest *ycfR* induction. Besides these three patterns of gene expressions, the most common gene expression alteration during this assay was associated with an unpredictable pattern, where the middle concentration of H_2O_2 caused the greatest gene expression alterations, either induction (*iroN*) or repression (*pocR*, *ompF* and *dps*). Recently, Mitosch et al. [30], using a dual-reported model, demonstrated the importance of timing of the gene expression during exposure of the microbial cell to an oxidative agent. Besides the importance of temporal gene expression response, this study points to a multifaceted effect of oxidative agent concentration on gene expression during oxidative stress, further indicating another dimension of cellular adaptation complexity to oxidative stress.

The comparative transcriptomic analyses revealed that the expression of 45% of the *S*. Enteritidis genome was altered at least two-fold upon exposure to H_2O_2. This finding showed that oxidative stress has a much greater impact on the physiology of this pathogen than previously anticipated [18,31,32]. Out of numerous biological functions altered by oxidative stress, the SUF mechanism of Fe-S cluster formation exhibited the greatest change. The Fe-S clusters are cofactors of proteins and they are involved in various critical metabolic processes including DNA replication, regulation, and repair, substrate uptake, respiration, and RNA modification [33]. In general, there are two Fe-S cluster formation systems, the ISC system and the SUF system [33]. In this study, the SUF system showed a significantly greater induction than the ISC system. This finding correlates with previous studies that pointed out that the ISC is a housekeeping system [34], while the SUF was shown to be an oxidative stress or Fe-limited inducible system [35]. Indeed, the transcriptomic analyses showed the

significant induction of numerous Fe acquisition mechanisms, including an iron/manganese transporter, ferrichrome, hemin, ferrioxamine, ferric coprogen and ferric-rhodotorulic acid uptake systems, then biosynthesis and uptake of salmochelin, enterobactin, as well as hydroxamate siderophores, clearly indicating a state of Fe-limitation in oxidatively stressed cells. These data showed the activation of the various Fe-acquisition systems in *S.* Enteritidis cells during oxidative stress, further suggesting their critical importance in cellular adaptation to oxidative stress.

Hydroxyl free radical, a product of the Fenton reaction, represents the most damaging compound during H_2O_2 treatment [36]. This highly reactive oxygen species interacts with numerous biomolecules further causing multiple effects on the treated prokaryotic cells [37]. The comparative transcriptomics analyses showed multifaceted responses of oxidatively treated cells including induction of not only the OxyR (*katG*, *ahpCF*) and the SoxRS (*soxR*, *soxS*, *sodA*, *sodC*), two oxidative stress regulons, but also stress responses involved in DNA and protein repairs, osmoprotection, multidrug efflux, phosphate and magnesium starvation. During the oxidative stress response, this human pathogen induced a robust DNA repair system, which included base repair (*deoBCD*), single base mismatch repair (*dinIG*), early double-strand break repair (*yebG*), multiple double-strand breaks repair (*recAN*) and DNA replication (*polB*, *dinP*). Also, the *dps* gene which encodes a multifunctional Dps protein was greatly induced. Dps non-specifically binds DNA, forming a highly stable Dps-DNA nucleoprotein complex which further protects DNA from the deleterious effects of ROS [38]. Similarly to DNA, protein repair systems were induced. The *ibpAB* genes, encoding small heat-shock proteins IbpA and IbpB, showed the greatest induction among various protein repair systems. Induction of genes encoding molecular chaperones (*dnaKJ*, *clpB*) and proteases (*clpA*, *pgtE*) indicates a tendency of proteins to aggregate or to undergo a process of denaturation during oxidative stress. Multifaceted responses of oxidatively treated cells was also reflected by induction of key genes associated with osmotic stress (*osmYC*, *envFR*), manganese starvation (*sitABCD*, *pagCDOK*, *mgtBC*), phosphate starvation (*phoEH*, *yjbA*, *phnTSUR*), multidrug efflux (*mdlAB*, *yhcQP*) and outer membrane modification (*wcaEFH*, *yncCJ*, *yjbAEHJ*). Adaptation of *S.* Enteritidis to oxidative stress was not only confined to the induced transcriptome but it also included the repressed transcriptome. The significant repression of the major small (*rpsUTPLGSOCFHNJQBR*) and large (*rplUKSJNXVBCDAEPLWYM*) units of ribosomal encoding genes indicates that oxidatively stressed cells activated a stringent response. The main purpose of a stringent response is saving cellular energy and precursors required for ribosomal synthesis. If cells continue to synthesize ribosomes, the majority of these organelles would end up being inactive due to the disruption of central metabolic pathways (i.e., lack of amino acids) and this would be an unnecessary and costly expenditure of resources [37].

Besides the alterations of a large pool of genes encoding various central metabolic and stress response pathways, the comparative transcriptome analyses revealed a clear division, during the H_2O_2 treatment, in the gene expressions associated with the genes encoding for motility (planktonic) and the adhesion genes (sessile) forms of life. The exposure of *S.* Enteritidis to oxidative stress had an extensive and significant positive effect on the expression of adhesion encoding genes, including fimbrial *stbABCD*, *fimHYWDF*, *sthB*, SEN4247; curli *csgABF*, and pilin *safBCD*, SEN1978 (type IV pilin), *yibEHG* and *yncC* operons and genes. At the same time, it negatively affected expressions of the major flagellar operons *flgHECFIGJDBMN* and *fliJMRLNKFQPOIHEBG* clearly indicating a switch from a planktonic to a sessile (i.e., biofilm) life forms of oxidatively treated cells. Recently, it was shown that a transition from planktonic to sessile life form in *S.* Enteritidis is followed by repression of the major flagellar operons and induction of numerous fimbrial, curli, and pili operons [22]. This transcriptional change is in agreement with the "swim-or-stick" theory, which postulates that motility and biofilm development are mutually-exclusive processes [39]. Our transcriptomic data indicate that oxidatively stressed cells transition from a planktonic to a biofilm form of life. Another important feature of oxidatively stressed cells was an extensive induction of salmonellae-essential virulence genes located on SPI 1, SPI 2, and SPI 5. Induction of core virulence genes involved genes encoding not only T3SS apparatus of the SPI 2 and translocon of SPI 1 but also numerous effectors (*sifAB*, *sseAbjbBFIE*, *iagB*,

sinHR, *sopE2*, *sicP*) essential for intracellular survival of this pathogen [40]. Fu and colleagues [18] studying the oxidative stress response of *S.* Typhimurium found that this organism repressed the synthesis of core virulence proteins encoded on SPI 1. Discrepancy between their and our results can be most likely explained by different experimental setups. While Fu and colleagues [18] exposed the cultures of *S.* Typhimurium to H_2O_2 at their mid-exponential growth phases (0.5 at OD_{600}) and harvested them at their early stationary growth phase (0.9 at OD_{600}). We started H_2O_2 treatment at the mid-exponential growth phase (0.4 at OD_{600}) and harvested the cultures at their still exponential growth phase (~0.5 at OD_{600}). Taken together, our experimental approach ensured that all transcriptional changes will be consequences of oxidative stress, while the approach employed by Fu et al., [18] reflects not only oxidative stress response but additional stress responses associated with the stationary growth phase of this organism [41,42].

5. Conclusions

This study employing a combination of incremental oxidative stress assays and the next generation sequencing approach revealed additional levels of the oxidative stress response complexity in non-typhoidal *Salmonella*. It was shown that the oxidative stress response is intricately linked with not only Fe-S cluster formation, iron homeostasis, DNA repair, and cell redox homeostasis but also with heat, osmotic, stringent, manganese and phosphate starvation stress responses as well as multidrug efflux and decreased passive influx of small hydrophilic molecules. In addition to these multifaceted cellular adaptation processes, it was shown that *S.* Enteritidis during oxidative stress simultaneously repressed the key motility encoding genes and massively induced the essential adhesion and salmonellae core-virulence encoding genes, crucial for the biofilm formation and host cell invasion. Based on these data, we hypothesize that the oxidative stress response of non-typhoidal *Salmonella* is closely associated with intracellular survival and that oxidative stress may act as a stimulus for eukaryotic cell invasion and formation of *Salmonella*-containing vacuoles. It has been already found that intracellular biofilm formation plays an important step in virulence of uropathogenic *Escherichia coli*, another facultative intracellular pathogen [43], suggesting that a similar approach can be explored by non-typhoidal *Salmonella*.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3921/9/9/849/s1, Figure S1. The effect of different H_2O_2 concentrations on the survivability of *S.* Enteritidis over 90 min. Error bars correspond to the standard deviations. Table S1. List of induced genes during the 3 mM H_2O_2 treatment of the *S.* Enteritidis ATCC 13076 strain compared to the control. Table S2. List of repressed genes during the 3 mM H_2O_2 treatment of the *S.* Enteritidis ATCC 13076 strain compared to the same H_2O_2 untreated strain.

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Conflicts of Interest: The authors declare no conflict of interest.

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