8-2017

Comprehensive Assessments of the Genetic Determinants in Salmonella Typhimurium for Fitness under Host Stressors: Oxidative Stress and Iron Restriction

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Comprehensive Assessments of the Genetic Determinants in *Salmonella* Typhimurium for Fitness under Host Stressors: Oxidative Stress and Iron Restriction

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Cell and Molecular Biology

by

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ABSTRACT

Salmonella is an intracellular pathogen that infects a wide range of hosts. The infected host utilizes reactive oxygen species (ROS) and iron-restriction to eliminate the pathogen. We used proteogenomics to determine the candidate genes and proteins that have a role in resistance of *S.* Typhimurium to H_2O_2. For Tn-seq, a highly saturated Tn5 library was grown *in vitro* under either 2.5 (H_2O_2L) or 3.5 mM H_2O_2 (H_2O_2H). We identified two sets of overlapping genes that are required for resistance of *S.* Typhimurium to H_2O_2L and H_2O_2H, and the results were validated via phenotypic evaluation of 50 selected mutants. The enriched pathways for resistance to H_2O_2 included DNA repair, aromatic amino acid biosynthesis (*aroBK*), Fe-S cluster biosynthesis, iron homeostasis and a putative iron transporter system (*ybbKLM*), flagellar genes (*fliBC*), H_2O_2 scavenging enzymes, and DNA adenine methylase. Proteomics revealed that the majority of essential proteins, including ribosomal proteins, were downregulated upon exposure to H_2O_2. A subset of proteins identified by Tn-seq were analyzed by targeted proteomics, and 70 % of them were upregulated upon exposure to H_2O_2. Further, we assessed genomic of *S.* Typhimurium under gradient iron-restricted conditions using Tn-seq. In addition to conditionally essential genes that mediate the pathogen survival under iron-restricted conditions, we found ROS-dependent essential genes. Based on this, we expand ROS-antibiotic mediated killing model, which asserts that bactericidal antibiotics induce ROS formation and ultimately contributes to cell death. We show that impairment of many essential genes with transposons, without antibiotic interference, induce ROS formation and the death of these mutants can be ceased through an iron chelator. Tn-seq reveals that one-third of *S.* Typhimurium essential genome are ROS-dependent, far beyond antibiotic targets, as they can grow very slowly in iron-restricted conditions. Interestingly, majority of antibiotic target genes are ROS-dependent. We propose that ROS-independent essential genes may be better targets for antibiotic development because the cells die immediately following the disruption of the

essential gene. This work expands our knowledge about mechanisms of *S.* Typhimurium survival in macrophages, the role of ROS in cell death following essential gene disruption, and provides novel targets for development of new antibiotics.

ACKNOWLEDGEMENTS

I am really grateful for my supervisor Professor Young Min Kwon, without him it would be impossible for me to get to this point. I am very thankful for his mentorship, encouragement, support, and training. Through his supervising and working at his lab, I learned countless things which will shape my science career to a right direction.

I would like thank my graduate research committee; Dr. Daniel Lessner, Dr. Ravi Barabote, and Dr. Jeffrey Lewis. Their advice, support, and help really appreciate it. Also, I would like thank the Cell and Molecular Biology program, particularly the director of program, Professor Douglas Rhoads for his endless support, without his support it would be very hard for me to pursue this degree.

My family, specifically my parents, are the main reason for me to get my degree and finish the study. Without my parent's support there was no way for me to write this acknowledgment here. Words can not describe how thankful I am to my mother, father, sisters and brothers, my sisters and brothers in law. They were all with me from the first day when I left home till I got to this point. I greatly appreciate their support and help.

I would like thank all lab members of Kwon Lab, specifically, the lab director, Dr. Tieshan Jiang. Special thanks to Dr. Rohana Liyanage at the mass spectrometry facility. I do thank all members at the poultry science and biological engineering department, particularly Donna Delozier, Dr. John Marcy, and Dr. Ronghui Wang.

I am very thankful for Paul Sagan and Denise Sagan, their family members and relatives for their support and companion through my staying in Fayetteville. Special thanks go to four Kurdish students in the University of Arkansas, Abdulla, Nawzar, Abdulkarim, and Zimnako.

So thankful to the Human Capacity Development Program, Kurdistan Regional Government (HCDP-KRG) for the partial fellowship. Finally, thank you UofA.

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PUBLISHED PAPERS

All of Chapter 2 comes from this paper, in review:

Karash S, Liyanage R, Qassab A, Lay J, Kwon Y. 2017. A Comprehensive Assessment of the Genetic Determinants in *Salmonella* Typhimurium for Resistance to Hydrogen Peroxide Using Proteogenomics. bioRxiv **115360.**

CHAPTER ONE

1.1 Introduction

Salmonella is an intracellular Gram-negative facultative pathogen that can infect a wide range of hosts. *Salmonella* classified into over 2,500 serovars based on the composition and structure of flagellar and lipopolysaccharide (Popoff et al., 2003), including typhoidal and non-typhoidal serovars (NTS). While typhoidal *Salmonella* is human restricted, which causes typhoid fever, NTS infects humans and animals. *Salmonella* infections frequently cause by consumption of contaminated foods or water and result in diverse clinical symptoms. NTS typically causes diarrhea, abdominal pain, fever, and may disseminate systemically via blood (Coburn et al., 2006). *Salmonella* serovars Typhimurium, Enteritidis, Newport, and Javiana account for about half of human infection cases that reported by health laboratories (Scallan and Mahon, 2012). Recently, outbreaks of multidrug-resistant *Salmonella* Heidelberg and Kentucky infections have increased (Gieraltowski et al., 2016; Tasmin et al., 2017). The common drug-resistant NTS infections are due to serovars Enteritidis, Typhimurium, Newport, and Heidelberg; there are 6,200 resistant culture-confirmed infections in the United States yearly (Medalla et al., 2017). Illnesses due to *Salmonella* infection are common in the world. NTS strains, including *Salmonella enterica* serovar Typhimurium, cause 1.2 million cases, 23,000 hospitalizations, and 450 deaths in the United States annually, this accounts for 11% of the total foodborne illnesses caused by different pathogens (Scallan et al., 2011). A study estimated that *Salmonella* accounts for 93.8 million infection cases of gastroenteritis which lead to 155,000 deaths globally per year (Majowicz et al., 2010). This pathogen is a constant threat to public health and food industry.

During the course of infection, *Salmonella* encounters a variety of host arsenals such as reactive oxygen species (ROS) and iron-restriction. However, *Salmonella* developed its own defense systems to overcome these host stressors, particularly in macrophages, where *S.*

Typhimurium faces NADPH oxidase–dependent killing (Vazquez-Torres et al., 2000). Derivatives of ROS are Hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot-}$), and the hydroxyl radical ($HO^{\cdot}$). The NADPH-dependent phagocytic oxidase reduces oxygen to superoxide anion which dismutates to H_2O_2 (Fang, 2004). Finally, H_2O_2 reduces to hydroxyl radical by ferrous iron through Fenton chemistry. The generated ROS can damage biomolecules, including DNA and Fe-S proteins (Imlay and Linn, 1998). H_2O_2 is an excellent oxidative stress agent for *in vitro* studies due to its stability. Nevertheless, there is no study on gene fitness measurement under H_2O_2 stress in bacteria using Tn-seq.

Iron is a cornerstone for numerous cellular metabolisms and serves as a cofactor for some proteins. Iron involves in respiration, tricarboxylic acid cycle, synthesis of metabolites, enzyme catalysis, and other biochemical reactions. Therefore, iron is considered a virulence factor and a crucial metal for survival of bacterial pathogens (Weiss 2002; Ganz and Nemeth, 2015). As iron accessibility is vital for intracellular *S*. Typhimurium virulence, the host uses a variety of mechanisms to sequester it from bacteria (Schaible and Kaufmann, 2005). Bacterial pathogens, including *Salmonella*, are employing aggressive acquisition processes to scavenge iron from hosts such as synthesize and excrete high-affinity iron chelators named siderophores (Raymond et al., 2003). Alternatively, it has been suggested that modulating host iron homeostasis may be a path to tackle multidrug-resistant intracellular bacteria (Kim et al., 2014). Experimentally, 2,2`-Dipyridyl (Dip) is the most commonly used iron chelator; it is a membrane permeable and a selective agent to chelate ferrous iron (Kohanski et al., 2007). Microarray has been used in bacteria to profile global transcriptional responses to iron limitation, using Dip as an iron chelator in *E. coli* (McHugh et al., 2003), *Shewanella oneidensis* (Yang et al., 2009), *Actinobacillus pleuropneumoniae* (Klitgaard et al., 2010), *Leptospira interrogans* (Lo et al., 2010), *Acinetobacter baumannii* (Eijkelkamp et al., 2011), and *S*. Typhimurium (Kim and Kwon, 2013). RNA-seq has also been applied for transcriptome

profiling in response to iron chelation by Dip in *Rhodobacter sphaeroides* (Remes et al., 2014) and *S.* Typhimurium (Kroger et al., 2013). All these studies exposed bacteria to a fixed concentration of Dip for a short time. However, bacteria encounter a variety of niches with different iron contents. Essential genome can not be identified directly using transcriptomics and our understanding of the genes in *S.* Typhimurium that are required for survival in iron-limited environments is not complete.

The development of antibiotic resistance in bacteria imposed a critical threat to human health. The extensive use of antibiotics fueled the problem. Therefor, development of new antimicrobial agents is urgently needed (Neu, 1992; Bush et al., 2011). The developed new antibiotics have to be smart in order to prevent the pathogen to become resistant for the agent. However, the molecular mechanism action of antibiotics is enigmatic. Classically, it has been suggested that antibiotic mainly kill the bacteria through inhibiting the essential pathways, however 10 years ago a study suggested a new model for the mode of antibiotic action (Kohanski et al., 2007). The model asserts that in addition to drug-target interaction, antibiotics ultimately generate ROS which damages biomolecules in the bacterial cell and causes death. This model, common antibiotic ROS-mediated killing, points that tricarboxylic acid cycle (TCA) and iron-sulfur clusters are the main players for ROS generation. Plentiful studies supported this model in the context of antibiotic-target interaction, however we know nothing regarding disruption of these essential genes and pathways via DNA transposon elements.

Genome-wide studies provided an important avenue for discovering functions of unknown genes in bacteria. Genome wide studies of bacterial gene expression have been shifting from microarray approach to RNA-sequencing (RNA-seq) employing next generation sequencing (Croucher and Thomson, 2010). Transposon sequencing (Tn-seq) is a combination of transposon mutagenesis in bacteria and next generation sequencing. Tn-seq is a powerful

technique to find gene fitness and function of unknown genes (Opijnen et al., 2010; Kwon, et al., 2016). Also, transcriptomics (RNA-seq) coupled with proteomics proved to be an excellent approach to quantify mRNA and protein abundances. Proteogenomics provides information about post-transcriptional, translational, and protein degradation (Vogel and Marcotte, 2012). To our best of knowledge there is no study combined Tn-seq with proteomics to study proteogenomics of a bacterium.

1.2 Objectives

In this study, at a system wide level, genomics of *S*. Typhimurium assessed under stress conditions. We used Tn-seq coupled with proteomics to identify the genes and proteins that are required for *S*. Typhimurium to resist H_2O_2 *in vitro*. We also identified ROS-dependent essential genes and genomic dynamics under gradient iron-restricted conditions in *S*. Typhimurium. We used *S*. Typhimurium and had the following main goals:

1. Develop an in-house Tn-seq method.
2. Identify the genes that are required for two concentrations of H_2O_2 resistance.
3. Utilizing untargeted and targeted proteomics to elucidate the proteins that are required for H_2O_2 resistance.
4. Identify the ROS-depended essential genes.
5. Genomic assessment under gradient iron-restricted conditions to find the genes that mediate the pathogen survival in theses stressors.

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CHAPTER TWO

A Comprehensive Assessment of the Genetic Determinants in *Salmonella* Typhimurium for Resistance to Hydrogen Peroxide Using Proteogenomics

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*Preprint, in review in Nature Scientific Reports
http://biorxiv.org/content/early/2017/05/27/115360

2.1 Abstract

Salmonella is an intracellular pathogen that infects a wide range of hosts and can survive in macrophages. An essential mechanism uses by the macrophages to eradicate *Salmonella* is production of reactive oxygen species. Here, we used proteogenomics to determine the candidate genes and proteins that have a role in resistance of *S.* Typhimurium to H_2O_2. For Tn-seq, a highly saturated Tn5 insertion library was grown *in vitro* under either 2.5 (H_2O_2L) or 3.5 mM H_2O_2 (H_2O_2H). We identified two sets of overlapping genes that are required for resistance of *S.* Typhimurium to H_2O_2L and H_2O_2H, and the results were validated via phenotypic evaluation of 50 selected mutants. The enriched pathways for resistance to H_2O_2 included DNA repair, aromatic amino acid biosynthesis (*aroBK*), Fe-S cluster biosynthesis, iron homeostasis and a putative iron transporter system (*ybbKLM*), flagellar genes (*fliBC*), H_2O_2 scavenging enzymes, and DNA adenine methylase. Proteomics revealed that the majority of essential proteins, including ribosomal proteins, were downregulated upon exposure to H_2O_2. A subset of proteins identified by Tn-seq were analyzed by targeted proteomics, and 70 % of them were upregulated upon exposure to H_2O_2. The identified candidate genes will deepen our understanding about mechanisms of *S.* Typhimurium survival in macrophages, and can be exploited to develop new antimicrobial drugs.

2.2 Introduction

Salmonella is a Gram-negative bacterium that infects humans and animals. *Salmonella enterica* has numerous serovars, which include typhoidal and non-typhoidal strains. In contrast to the typhoidal salmonellae which are human restricted pathogens, the non-typhoidal salmonellae (NTS), serovar Enteritidis and Typhimurium, are able to infect a wide range of hosts, causing gastroenteritis[1]. The NTS strains, including *Salmonella enterica* serovar Typhimurium, account for 11% (1.2 million cases) of the total foodborne illnesses caused by different pathogens in the United States[2]. It has been estimated that *Salmonella* is responsible for 93.8 million cases of gastroenteritis, leading to 155,000 deaths worldwide annually[3]. The pathogen remains a continuous threat to the food safety, and public health.

To initiate an infection and survive inside the host, *Salmonella* needs to overcome a myriad of host defense mechanisms. As *Salmonella* reaches the intestine and breaches the epithelial tissue, it enters the macrophages and activates different virulence strategies in order to survive and replicate in them[4]. An essential mechanism uses by the phagocytes to kill and eradicate *Salmonella* is production of reactive oxygen species (ROS). Hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot -}$), and the hydroxyl radical ($HO^{\cdot}$) are derivatives of ROS. The short-lived $O_2^{\cdot -}$, produced by the NADPH-dependent phagocytic oxidase, quickly dismutates into H_2O_2, which diffuses across semipermeable bacterial cell membranes. Eventually, Fe^{2+} reduces H_2O_2 to $HO^{\cdot}$ via the so called Fenton Reaction[5-7]. The ROS, including H_2O_2, can damage DNA, iron-sulfur cluster-containing proteins, and other biological molecules in the bacterial cells[8-10].

Numerous genetic factors and proteins that are important for resistance of *S.* Typhimurium to H_2O_2 have been discovered and the underlying mechanisms have been explored[11, 12]. A various approaches and techniques have been employed to study global

response of *Salmonella* or related bacteria to H_2O_2 *in vitro* as a model system to simulate the bacterium's response to ROS in phagocytic cells: (i) Two-dimensional gel electrophoresis identified H_2O_2-induced proteins in *Salmonella*[13], (ii) DNA microarray identified H_2O_2 induced genes in *E. coli*[14], and (iii) RNA-seq identified H_2O_2 induced genes in *Salmonella*[15]. Yet, the factors required for fitness under the given condition cannot be identified with high confidence based on the analysis of transcriptomics or proteomics data[16]. Microarray-based tracking of random transposon insertions was used to identify numerous genes in *Salmonella* that are required for survival in mice and macrophages[17-18]. However, the genetic factors responsible for resistance to ROS cannot be sorted out among all of the genetic factors identified in the study that are required for fitness in the presence of multiple host stressors.

To shed more insights into the underlying mechanisms of *Salmonella* resistance to H_2O_2, more direct approach linking the gene-phenotype relationships in a genome-wide scale would be necessary. Tn-seq is a powerful approach to allow direct and accurate assessment of the fitness requirement of each gene on the entire genome of a prokaryotic organism[19]. In Tn-seq method, a saturated transposon insertion library (input) is exposed to a selective condition, and the mutant population altered through the selection (output) is recovered. Then, the genomic junctions of the transposon insertions are specifically amplified and sequenced from both input and output pools by high-throughput sequencing. The gene fitness can be obtained by calculating the change in relative abundance of the sequence reads corresponding to each gene in the entire genome between the two pools. Tn-seq has been employed to assign gene functions to *Salmonella* genomes in numerous studies: (i) Previously, our lab identified conditionally essential genes that are required for growth in the presence of bile, limited nutrients, and high temperature[20], (ii) The genes required for intestinal colonization were identified in chickens, pigs, and cattle[21], (iii) Candidate essential genes and genes contributing toward bile resistance were identified[22], (iv) Core conserved genes for growth in rich media

were identified in serovars Typhi and Typhimurium[23]. In addition to Tn-seq, electrospray ionization liquid chromatography tandem mass spectrometry (ESI-LC-MS/MS) is a powerful approach for identifying and quantifying proteins in a large scale. The system-wide protein regulation can be determined using mass spectrometry signal intensities of tryptic peptides obtained from two different culture conditions[24]. The post-translational modification in proteins can be revealed by using proteomic analysis[25]. Many studies took advantage of proteomic analysis of *Salmonella*. However, to the best of our knowledge, this study is the first to investigate proteogenomics of a bacterium by combining Tn-seq and proteome analysis simultaneously to the same stressor.

In this work, we used Tn-seq method and proteomic analysis in combination to determine system-wide responses of *S.* Typhimurium to two different concentrations of H_2O_2 (H_2O_2L and H_2O_2H). We obtained a comprehensive list of 137 genes that are putatively required for the resistance of *S.* Typhimurium 14028 to H_2O_2. The role of 50 selected genes in resistance to H_2O_2 were determined by phenotypic evaluation of the individual deletion mutants. Also, we identified a set of 246 proteins that are differentially expressed in response to H_2O_2, using data-dependent acquisition (DDA) proteomics, which are largely overlapped with the genes identified by Tn-seq; targeted proteomics showed 70% of the proteins identified by Tn-seq were upregulated by H_2O_2. In addition to the genes of *S.* Typhimurium previously known to be important for resistance to H_2O_2, we identified approximately 80 genes that have not been previously associated with resistance to oxidative stress. The results of this study highlighted that the genes in aromatic amino acid biosynthesis, *aroB* and *aroK*, and iron homeostasis, *ybbK*, *ybbL*, and *ybbM*, are crucially important for growth fitness under H_2O_2 stress. The identified candidate genes will expand our understanding on the molecular mechanisms of *Salmonella* survival in macrophages, and serve as new antimicrobial drug targets.

2.3 Results and Discussion

2.3.1 The H_2O_2 concentrations and the selections of Tn5 library

First, we sought to determine the growth response of wild type *S.* Typhimurium 14028 cells in LB media containing varying concentrations of H_2O_2. The wild type cells were grown in LB media that contain different concentrations of H_2O_2 in 96-well plates. After evaluating the growth rates for the cultures, 2.5 and 3.5 mM H_2O_2 were chosen for Tn-seq selections in our study, and termed H_2O_2L and H_2O_2H, respectively. In comparison to *Salmonella* grown in LB media with no H_2O_2, H_2O_2L and H_2O_2H reduced the growth rates by 10% and 28%, respectively (Fig 2.1A). The lag time increased by a 5.7-fold (0.5 vs. 2.9 hr), and an 11-fold (0.5 vs. 5.6 hr) in H_2O_2L and H_2O_2H, respectively. The maximum OD_{600} decreased by only 1% for the H_2O_2L and 2% for the H_2O_2H in comparison to LB media (Fig 2.1A).

For the selection of Tn5 library, 20 ml cultures in 300 ml Erlenmeyer flasks containing LB, H_2O_2L, or H_2O_2H were inoculated with the same Tn5 library at the seeding CFUs of the library at 3.5×10^6. This seeding level provided ~10 CFUs for each Tn5 insertion mutant in the library. The cultures were grown until the mid-exponential phase, in which the CFUs reached 1.17×10^8 (SE 0.01×10^8). It required 7.5 and 9.2 h to reach the cell density as measured by optical density for H_2O_2L and H_2O_2H, respectively, in contrast to 5 h for LB medium (Fig 2.1B). We observed some differences in growth responses between the cultures in a 96-well plate and in a 300-ml Erlenmeyer flask. The optical density readings by the plate reader was different in comparison to those by Bio-photometer that we used to measure optical density of the culture in the flask. As a result, the growth curve in Fig 2.1A which was based on 96-well plate reader, dose not match exactly with the time required for the Tn5 library to reach the target mid-exponential phase in the flask cultures. In addition, we observed that the H_2O_2 is stable in LB media free of *Salmonella* during the window of time used for the library selection process (Fig. S2.1), which was also supported by Bogomolnaya et al.[26].

2.3.2 Preparation of Tn5-seq amplicon library

The *Salmonella* mutants were generated by using the delivery plasmid pBAM1 via conjugation. A total of 325,000 mutant colonies were recovered from 50 plates. Each mutant contained a single random insertion of Tn5 transposon in the chromosome or plasmid according to DNA sequencing of Tn5-junction sequences for a small set (n = 71) of randomly selected Tn5 mutants. We found a significant portion (~20%) of the mutants in the library that were not genuine Tn5 insertions, but the mutants generated as a result of pBAM1 integration into chromosome as determined by their ability to grow in the presence of ampicillin. To prevent the Illumina sequencing reads of being wasted on sequencing Tn5 junctions from these cointegrants, we digested genomic DNA of the input and output libraries with PvuII, which digests immediately outside the inverted repeats on both sides of Tn5. The digested DNA was then used to prepare Tn-seq amplicon library as described in Materials and Methods. Our Tn-seq data analysis indicated that our strategy of removing the DNA sequences originating from cointegrants was effective because only 0.55% of the total HiSeq reads corresponding to Tn5-junctions matched to pBAM1. It should be possible to remove them completely by ensuring complete digestion of genomic DNA with PvuII. The method for Tn-seq amplicon library we developed and used in this study has multiple advantages over other Tn-seq protocols, because our method requires only 100 ng of the genomic DNA, and the whole process can be completed in a day[27]. When the extension step in the protocol was performed using a conventional 20 nucleotide primer, and the final products of exponential PCR were separated on agarose gel electrophoresis, even the negative controls (the wild type genomic DNA or mutant library genomic DNA without linear extension) showed smear patterns of nonspecific background amplification. However, when dual priming oligonucleotide (DPO) primer was used in place of the conventional primer for linear extension, non-specific background amplification was completely disappeared. Therefore, we adopted the DPO primer in linear extension step for all

library samples in this study. Then, the single-stranded extension products were C-tailed, and used as templates for the exponential PCR step using nested primer specific to Tn5 and poly G primer that contain Illumina adapter sequences along with sample index sequences (Fig. S2.2). The final PCR products were separated on an agarose gel, and the fragments within the range of 325-625 bp were gel-purified. After pooling of multiple samples, the combined library was sequenced on a HiSeq 3000.

2.3.3 Summary of Tn-seq DNA analysis

After de-multiplexing and C-tail trimming of all sequence reads, ~72 million reads of Tn5- junctions with mean read length of 94 bp were obtained. The number of the reads mapped to the complete genome of *S.* Typhimurium 14028 were ~25, 15, and 19 million for LB, H_2O_2L, and H_2O_2H, respectively. The number of unique insertions on the chromosome were 125,449 in the input library, excluding the plasmid (Table S2.1). On average, Tn5 was inserted in every 39 bp. Number of raw reads per open reading frame (ORF) for H_2O_2L was plotted over the corresponding number of H_2O_2H, which yielded an R^2 of 0.91, indicating the mutants in the input library quantitatively responded in a similar way for both H_2O_2L and H_2O_2H as expected (Fig. S2.3). The insertions were mapped to 5,428 genes or 8,022 genes/intergenic regions. Interestingly, the ORF STM14_5121, which is 16.7 kbp long, had the highest number of insertions (~700 insertions) and reads (0.25 M).

2.3.4 Comparison of various bioinformatics pipelines for Tn-seq data analysis

We used 3 different Tn-seq analysis tools to identify the genes and compare the results across the methods with the goal of comprehensive identification of "all" genes required for resistance to H_2O_2. The first tool, ARTIST[28], created small non-overlapping genomic windows of 100 bp and the reads from each window were arbitrarily assigned into the middle of the window. The default normalization script of the tool was used. Then, the relative proportions

of insertion sites in the output library versus the input were tabulated. Mann-Whiney *U* (MWU) test was used to assess the essentiality of the locus. To consider a gene/intergenic region conditionally essential for growth in the presence of H_2O_2, *p* value had to be ≤ 0.05 in 90 of the 100 conducted MWU tests. Subsequently, 20 genes and 1 intergenic region were identified for H_2O_2L and 4 genes for H_2O_2H (Table S2.2). We speculate the reason that more genes were identified for H_2O_2L in comparison to H_2O_2H, was partially due to the lower number of total reads of H_2O_2L as compared to H_2O_2H, even though the read numbers of H_2O_2L was normalized to those of the input.

The second tool, Tn-seq Explorer[29], counted insertions in overlapping windows of a fixed size. Using a 550 bp window size, each annotated gene was assigned an essentiality index (EI) which is determined mainly based on the insertion count in a window in this gene. The bimodal distribution of insertion counts per window divided the essential genes to the left and the non-essential genes to the right. To find conditional essential genes, the EI of the output was subtracted from the EI of the input. The genes with negative ΔEI were ranked based on the change in read fold change (Log2 (H_2O_2L or H_2O_2H/Input)). We found 114 consensus genes between H_2O_2L and H_2O_2H that had at least four-fold reduction in H_2O_2H read counts as compared to the input. The four-fold reduction (Log2FC = -2) threshold was chosen based on our validation study of Tn-seq data by single mutant assays (Table S2.2).

The third tool, TRANSIT[30], determined read counts of genes in the input and output library. The differences of total read counts between the input and outputs were obtained. The insertion sites were permutated for a number that is specified by the user (we used 10,000 sample). This sampling for each gene gave difference in read counts. The *p* value was calculated from the null distribution of the difference in read counts. We identified 8 and 21 genes for the H_2O_2L and H_2O_2H, respectively, using a *p* value ≤ 0.05 (Table S2.2).

The combined list of the genes identified by the 3 Tn-seq analysis tools for both H_2O_2L and H_2O_2H included 137 genes (Table S2.2). All of the genes on this list are expected to have a role in conferring resistance to H_2O_2 and allow *Salmonella* to survive and replicate in the presence of H_2O_2 *in vitro*. Of the 21 genes identified by TRANSIT, 19 of these genes were also identified by Tn-seq Explorer, but only 3 out of this 21 were identified by ARTIST. The 19 genes were *hscA*, *rbsR*, *fepD*, *efp*, *oxyR*, *polA*, *ybaD*, *aroD*, *ruvA*, *xthA*, *dps*, *aroB*, *uvrD*, *tonB*, *uvrA*, *aroK*, *ybbM*, *lon*, and *proC*. Two genes, *fepD* and *xthA*, were identified by the all 3 methods and for both conditions.

The 3 Tn-seq analysis tools are very valuable for Tn5 data analysis, but each tool has its own advantages and disadvantages. For ARTIST, (i) the user must know how to run scripts in Matlab software, (ii) the analysis is very slow on a personal computer with the HiSeq data, (iii) it has only one method for normalization, but (iv) it can search for essentiality in the intergenic regions. For Tn-seq Explorer, (i) there is no data normalization, and (ii) prediction on small genes is prone to be inaccurate, but (iii) its very user-friendly and runs fast. For TRANSIT, (i) the user should have some knowledge on running scripts on terminal, (ii) it may need some modification in its Python script according to the way the library was prepared for sequencing, and (iii) a few software packages should be installed on the computer as TRANSIT pre-requisites, but (iv) it does have 6 different methods for data normalization and it runs very fast on a personal computer. Although ARTIST and Tn-seq Explorer are very useful tools for Tn-seq data analysis, we prefer using TRANSIT in our future data analysis for conditionally essential genes. In the following sections, we continued the downstream analysis mainly based on the 137 genes that include all of the genes identified by all 3 methods.

2.3.5 The enriched pathways for resistance to H_2O_2

In order to categorize the identified genes that are required for *Salmonella* resistance to

the H_2O_2, the 137 genes were subjected to pathway enrichment analysis using DAVID Bioinformatics Resources 6.7, NIAID/NIH[31]. A total of 15 KEGG pathways[32] were recognized for 69 genes on the list. The enriched pathways include homologous recombination (*ruvC*, *polA*, *ruvA*, *ruvB*, *priB*, *recA*, *recR*, *holC*, *holD*, *recC*, *recG*), nucleotide excision repair (*uvrD*, *polA*, *uvrA*, *uvrC*), mismatch repair (*dam*, *uvrD*, *holC*, *holD*), RNA degradation (*pnp*, *hfq*, *ygdP*), purine and pyrimidine metabolism (*apaH*, *polA*, *pnp*, *arcC*, *spoT*, *holC*, *holD*, *cmk*, *dcd*, *pnp*), phenylalanine, tyrosine and tryptophan biosynthesis (*aroD*, *aroB*, aroA, *aroK*, *aroE_2*), arginine and proline metabolism (*proC*, *arcC*), glycolysis and gluconeogenesis (*crr*, *pgm*, *tpiA*), oxidative phosphorylation (*atpG*, *atpA*, *cydA*), DNA replication (*polA*, *holC*, *holD*), and flagellar assembly (*fliJ*, *fliD*, *flhD*, *fliC*). Since KEGG was not able to recognize many genes on the list, we used SP_PIR_Keywords of functional categories, which recognized majority of the genes and categorized them into 55 functional categories (Table S2.3), excluding 15 uncharacterized genes (ORFs). Among these categories were stress response (*rpoE*, *lon*, *dnaJ*, *hfq*, *yaiB*), iron (*dps*, *entD*, *iscA*, *yjeB*, *yhgI*), and transcription regulation (*rcsA*, *oxyR*, *rpoE*, *yjeB*, *arcA*, *argR*, *rbsR*, *rpoS*, *fadR*, *rcsB*, *furR*, *flhD*).

2.3.6 Validation of Tn-seq results using individual mutants

For the selected 50 genes among the 137 genes identified by Tn-seq, the growth phenotype was determined using individual single deletion mutants in LB, H_2O_2L, and H_2O_2H. The genes were considered to play a role in resistance to H_2O_2, if (i) lag phase time increased, (ii) growth rate reduced or (iii) maximum OD_{600} decreased in the presence of H_2O_2 in comparison to the wild type strain grown in the same conditions. Of the 50 single deletion mutants, 42 mutants were shown to have a role in resistance to H_2O_2 (Fig. 2.2 and Table S2.4). One gene, *yhaD*, was identified by all 3 analysis tools, but it did not show the expected phenotype. The *fliD* was also identified by ARTIST, but did not show any phenotype distinguishable from the wild type. The remaining 6 genes that did not show the phenotype

was identified by Tn-seq Explorer. Based on the results of the individual mutant assay, we conclude that 84% (42/50) of the genes identified by the Tn-seq analysis and tested using single deletion mutants have a role for resistance to H_2O_2. These results indicate that our Tn-seq analysis identified the genes in *S.* Typhimurium that are required for the wild type level resistance to H_2O_2 with high accuracy.

2.3.7 Proteomics of H_2O_2 response

With ESI-LC-MS/MS in data-dependent acquisition (DDA) mode, the protein regulation was determined using MS1 filtering technique that skyline software offers[33]. It uses signal intensities of tryptic peptides derived from the proteins of wild type strain grown in the presence of H_2O_2 in comparison to the control (LB). As described in Materials and Methods section, trypsin digestion of the protein extracts under different conditions generates tryptic peptides that are uniquely related to individual proteins. Tryptic peptides separated by liquid chromatography from the complex samples were first subjected to simple mass measurement (MS1) followed by intensity dependent fragmentation of these peptide ions to produce sequence specific fragment ions by collision-induced dissociation (MS/MS). Tryptic peptides were then identified using these sequence specific fragment ions via MASCOT database search software[34], where the sequence specific fragment ions were matched to the proteins in *S.* Typhimurium 14028S reference proteome database[24, 35]. This method of protein analysis is normally referred to as data dependent analysis (DDA). At the beginning of data analysis, the H_2O_2L and H_2O_2H data were compared to LB separately, however it turned out that comparison was not sensitive enough to differentiate between H_2O_2L and H_2O_2H conditions. Hence, the data of H_2O_2L and H_2O_2H were combined for analysis in comparison to LB. We identified 1,104 proteins of *Salmonella* for the 3 conditions (Table S2.5); of these, 246 proteins were differentially expressed in response to H_2O_2 with p values ≤ 0.05 and 90% CI. Proteomics analysis showed that 121 and 125 proteins were upregulated and downregulated in response to

stress by H_2O_2, respectively. Since Tn-seq revealed genetic requirements for fitness under the selection conditions, the identified genes are expected to express corresponding proteins under the conditions to perform their cellular functions. Often the proteins required for fitness under a given condition are overexpressed under the condition, but it may not be the case for some proteins. In this study, we had a unique opportunity to comparatively analyze both Tn-seq and the MS data to understand the relationship between genetic requirements and changes in expression level under the condition of interest, which was H_2O_2 in this study. We also obtained the list of essential genes based on our Tn-seq data, which could not tolerate insertions by definition, and if we were not certain about essentiality of a gene from our Tn-seq data, the gene was searched for essentiality in the previously reported list of *Salmonella* essential genes[22]. The comprehensive list of essential genes allowed us to study any correlation between the essentiality and the changes in protein expression. Among the 246 proteins, there were 78 essential and 168 non-essential proteins. Among the 78 essential proteins, 25 were upregulated whereas 53 were downregulated. On the contrary, the majority (n = 96) of the detected non-essential proteins were upregulated, while 72 non-essential proteins were downregulated. To further examine the quantitative relationships closely, 64 genes/proteins identified by both methods (Table S2.5) were focused on. Among the 64 genes/proteins, 57 genes showed negative Log2FC based on Tn-seq data, and 41 proteins among the 57 were upregulated at protein level. However, only 12 proteins had p values of ≤ 0.05 (AhpC, ArcA, Crr, DksA, FliC, IcdA, OxyR, Pgm, RecA, RpoS, SlpA, and WecE).

Using KEGG pathway analysis, 150 proteins among the 246 were enriched in 21 pathways (Table S2.6). Interestingly, of the all 59 30S and 50S ribosomal proteins in *S.* Typhimurium, 37 of these proteins (63%) were downregulated in response to H_2O_2. Moreover, of the 8 identified proteins in TCA cycle, 6 proteins were downregulated, including 2 essential proteins.

Although DDA method can be used to search for all proteins in a complex sample, it is prone to miss identification of important proteins due to the fact that fragmentation of tryptic peptides from these proteins may not be triggered as a result of lower peptide ion intensities compared to the threshold set. To quantify proteins expressed for the genes identified by Tn-seq more precisely and accurately, we used targeted-proteomic approach by employing liquid chromatography coupled with triple quadrupole mass spectrometry (LC-QQQ-ESI-MS). Here, tryptic peptides of the protein were targeted for fragmentation (MS/MS) independent of their intensities, as described in Materials and Methods, and the observed sequence specific fragment ion intensities from three unique tryptic peptides were utilized for protein quantitation. Of the 137 Tn-seq identified genes, we selected 33 genes to quantify their proteins in response to H_2O_2 by using targeted proteomics (Table S2.5). Interestingly, 23 (70%) of the 33 tested proteins were upregulated in response to H_2O_2. This shows a good agreement between the results of the Tn-seq and the targeted proteomics.

2.3.8 Aromatic amino acid biosynthesis genes are required for H_2O_2 resistance

Interestingly, our Tn-seq data revealed that the aromatic amino acid biosynthesis and metabolism pathway play a role in conferring resistance in *Salmonella* to H_2O_2 (Fig. 2.3A and 2.3B). Five genes, *aroB*, *aroD*, *aroE_2*, *aroK*, and *aroA* in the aromatic amino acid biosynthesis pathway were identified by Tn-seq, and the fitness of the mutants were significantly reduced in the presence of H_2O_2. To confirm this, 4 of these genes were evaluated using individual mutant assays. The *Salmonella aroK* mutant showed the strongest phenotype, because it failed to grow in the presence of H_2O_2L or H_2O_2H during 24 h incubation time. Also, the *aroB* mutant exhibited a strong phenotype, significantly extending lag phase for both H_2O_2 conditions. The *aroE_2* mutant also exhibited an extended lag time, but the *aroA* mutant did not show any difference in growth phenotype in the presence of H_2O_2. In addition, targeted-proteomics also showed that all these 5 proteins were upregulated in response to H_2O_2 (Fig.

2.3C and Table S2.5).

ROS damages a variety of biomolecules via Fenton reaction, which consequently lead to metabolic defects, specifically auxotrophy for some aromatic amino acids 10. *E. coli* mutants that lack superoxide dismutase enzymes are unable to grow *in vitro* unless the medium are supplemented with aromatic (Phe, Trp, Tyr), branched-chain (Ile, Leu, Val), and sulfur-containing (Cys, Met) amino acids[36]. We identified the genes in the aromatic amino acid biosynthesis pathway that are critically important for resistance to H_2O_2. In this pathway, *aroK* catalyzes the production of shikimate 3-phosphate from shikimate, which consequently leads to the production of tryptophan, phenylalanine, tyrosine and some metabolites from the chorismate precursor in *E. coli*. Further, *aroK* mutant in *E*. coli displays increased susceptibility to protamine, a model cationic antimicrobial peptide. It has been suggested that resistance to protamine is probably due to the aromatic metabolites and product of *aroK* gene, which act as a signal molecule to simulate the CpxR/CpxA system and Mar regulators[37]. In our Tn-seq data, *cpxR*/*cpxA* and *marBCRT* were in the list of non-required genes, but the proteomics data indicated that CpxR was upregulated. Also, *aroK* mutant in *E. coli* is resistance to mecillinam, a beta-lactam antibiotic specific to penicillin-binding protein 2. It has been concluded that the AroK has a secondary activity in addition to the aromatic amino acid biosynthesis, probably related to cell division[38]. In addition, *aroK* gene presents a promising target to develop a non-toxic drug in *Mycobacterium tuberculosis* because *aroK* is the only *in vitro* essential gene among the aromatic amino acid pathway genes and blocking *aroK* kills the bacterium *in vivo*[39]. Moreover, *aroK* gene plays a general role in *S.* Typhimurium persistence in pigs[40]. The *aroB* is another gene in the pathway that was identified by Tn-seq, which encodes 3-dehydroquinate synthase in the Shikimate pathway, aromatic amino acid biosynthesis pathway. *Salmonella* lacking *aroB* showed a strong growth defect in the presence of H_2O_2. When this mutant grown in the presence of H_2O_2, it increased the lag phase time by a 114-fold for the H_2O_2L and a 347-

fold for the H_2O_2H as compared to the mutant grown in absence of H_2O_2. *S.* Typhimurium mutant lacking the *aroB* gene is attenuated in BALB/c mice[41]. In addition to *aroK* and *aroB*, *aroE_2* was also shown to be important for resistance to H_2O_2, because deletion of the *aroE_2* reduced the growth rate by 35% in the presence of H_2O_2 and increased the lag phase time, too. All these 3 genes in this pathway are required for systemic infection of *Salmonella* in BALB/c mice in a more recent study[18]. We observed that there was a strong correlation between the fitness based on Tn-seq data, growth rates measured by individual mutant assays, and upregulation of their proteins quantified via targeted proteomics. This demonstrates the power of proteogenomic approach in discovering and characterizing the genes that are required for growth under a specific condition.

2.3.9 The *ybbM*, *ybbK*, and *ybbL* have a role in H_2O_2 resistance

The mutants with single deletion in each of *ybbK*, *ybbL*, and *ybbM* genes on the same pathway showed a strong phenotype against the activity of H_2O_2 in a dose-dependent manner. Based on Tn-seq data, the fitness of *ybbM* was -1.16 and -1.79 for H_2O_2L and H_2O_2H, respectively (Fig. 2.4A). The *ybbM* mutant demonstrated decreased growth rate by 38% for H_2O_2L and 100% for the H_2O_2H as compared to the mutant grown in the absence of H_2O_2. This mutant also increased the lag time by a 126-fold and a 267-fold for H_2O_2L and H_2O_2H, respectively (Fig. 2.4B). Also, the fitness score of *ybbK* was -0.92 for H_2O_2L and -1.81 for H_2O_2H. The *ybbK* mutant showed decrease of growth rate for H_2O_2L and H_2O_2H by 85% and 95%, respectively. The deletion increased the lag phase by a 46-fold and a 114-fold in the presence of H_2O_2L and H_2O_2H, respectively (Fig. 2.4B). Moreover, the fitness of *ybbL* mutant was -1.05 and -1.73 for H_2O_2L and H_2O_2H, respectively. Deleting the *ybbL* in *Salmonella* led to decrease in growth rate by 27% for H_2O_2L and 92% for H_2O_2H. The lag phase time for this mutant increased by a 22-fold and a 33-fold for H_2O_2L and H_2O_2H, respectively. In addition, YbbM, YbbL, and YbbK proteins were upregulated in response to H_2O_2; YbbM was the most

upregulated protein among the 3 proteins (1.46-fold), followed by YbbL (1.29-fold), and YbbK (1.25-fold) (Fig. 2.4C and Table S2.5). The fitness scores of the Tn-seq of these 3 genes are correlated strongly with the growth rate, lag time of their respective mutants, and upregulation of their proteins. As the number of reads depletes after the selection for a mutant, (i) there was more reduction in growth rate, (ii) the mutant stays longer in the lag phase, and (iii) the protein expression elevates. These observations clearly point to their role in conferring resistance to the H_2O_2-mediated stress. These genes were described in the *Salmonella* reference genome as follows: *ybbM*, putative YbbM family transport protein, metal resistance protein; *ybbK*, putative inner membrane proteins; *ybbL*, putative ABC transporter, ATP-binding protein YbbL. To the best of our knowledge, there is only one published study on the *ybbM* and *ybbL*[42]. Based on their findings, YbbL and YbbM have a role in iron homeostasis in *E. coli* and are important for survival when the bacterium was challenged with 10 mM H_2O_2 for 30 min; this putative ABC transporter transports iron and lessens ROS species formation that generates via H_2O_2. In this study, we identified an additional gene, *ybbK*, in the same pathway as the gene required for resistance to H_2O_2, strongly establishing the role of these 3 genes in resistance to H_2O_2.

2.3.10 The H_2O_2 scavenging and degrading genes

Salmonella employs redundant enzymes to degrade or scavenge ROS. The *katE*, *katG*, and *katN* genes encode catalases, which are involved in H_2O_2 degradation. The *ahpCF*, *tsaA*, and *tpx* genes encode peroxidases, which scavenge H_2O_2. The *sodA*, *sodB*, *sodCI*, and *sodCII* genes encode superoxide dismutases and these enzymes specifically scavenge O_2[11, 12, 43-45]. However, none of these were present in the list of genes identified by Tn-Seq. Even though *katE*, *katG*, *ahpC*, *sodA*, *sodCI*, and *sodCII* showed reduced fitness, they did not meet the statistical threshold. However, the proteomics data indicated that AhpC (1.48-fold), SodB (1.46-fold), and TpX (1.39-fold) were upregulated in the presence of H_2O_2 (Table S2.5) and

KatG was also upregulated, but its *p* value was 0.054. This reveals that these 4 proteins were the most important enzymes for H_2O_2 resistance under our experimental conditions. *Salmonella* containing an *ahpC* promoter-gfp fusion shows that expression of the *ahpC* is regulated by ROS that is generated from macrophages or exogenous H_2O_2 and the response to H_2O_2 is in a dose-dependent manner[46]. *Salmonella* mutant that lacks *katE*, *katG*, or *ahpCF* can degrade micromolar concentrations of H_2O_2. However, *Salmonella* mutant that has deletions in the all 5 genes, *katE*, *katG*, *katN*, *ahpCF* and *tsaA* (HpxF), cannot degrade H_2O_2, is unable to proliferate in macrophages, and show reduced virulence in mice[11]. This emphasizes that *ahpC*, *sodB*, and *tpx* may be the primary players in scavenging and degrading H_2O_2 in our experiment. Why Tn-seq did not detect any of these genes, while proteomics detected only these 3 proteins among others? It may reflect the functional redundancy in the genetic network that prevented single deletions in one of these genes from exhibiting fitness defect. Alternatively, when these mutants were grown together with all other mutants in the library, the functional protein lacking in one mutant due to Tn5 insertion could have been compensated by the other mutants in the library.

In addition to these genes, *oxyR* was detected by Tn-seq (Fig. 2.1C) and DDA proteomics. The *oxyR* was identified by all 3 analysis methods of Tn-seq data and it was on the top of the list, indicating a severe fitness defect of the mutant. The *oxyR* gene encodes H_2O_2 sensor and transcription factor, which mediates protection against ROS. The *katG* and *ahpCF* are regulated by OxyR, peroxide response regulator[13, 14]. Although *Salmonella* OxyR regulon is induced in the *Salmonella*-containing vacuole in macrophage, the *oxyR* mutant was virulent in a BALB/c mouse and can grow well in human neutrophils *in vitro*[47, 48]. The fitness of *oxyR* mutant was reduced for both H_2O_2L and H_2O_2H with the respective fitness score of -4.96 and -5.94. *Salmonella oxyR* mutant exhibited a growth rate reduction by 24% and 40% for H_2O_2L and H_2O_2H, respectively. Comparing this reduction in growth rate to the other mutants such as

rpoS or *aroK*, we observed that the *oxyR* mutant did not show severe phenotype and the mutant escaped from the lag phase easily. Moreover, our targeted proteomics indicated that the OxyR was not upregulated significantly. Further studies are needed to uncover the exact role of OxyR in response to ROS. However, previous studies implied that OxyR plays an essential role in resistance to H_2O_2 by regulating other proteins. OxyR induces Dps in *E. coli*, a ferritin-like protein that sequesters iron[49]. Sequestering of iron impairs the Fenton reaction, which consequently provides protection against ROS and reduces the damage of biomolecules. The *dps* gene was identified by the Tn-seq and its fitness score was -2.48. However, the Dps protein was downregulated based on the DDA proteomic analysis. To confirm this unexpected finding, we conducted the proteomic assay twice and each time with at least 4 technical replicates, but the Dps protein was significantly downregulated with $p = 0.001$. Further, the targeted-proteomics demonstrated the same result, pointing to the downregulation of Dps in response to H_2O_2. This is contrary to the previously reported works on Dps in *Salmonella* and the reason for the discrepancy is unclear.

2.3.11 DNA repair system is important for H_2O_2 resistance

The imposed exogenous H_2O_2 activates DNA repair system in *Salmonella* in order to repair or eliminate the damage that occurred on the nucleotides. The *E. coli* RecA protein repairs double-strand DNA lesions through recombination[50]. In our Tn-seq analysis, the fitness score of this mutant was -5.36 for both concentrations, and in proteomics, the RecA was upregulated (1.79-fold). *Salmonella recA* mutant decreased the maximum OD_{600} by 16% for H_2O_2L and 22% for H_2O_2H as compared to the same mutant grown in LB. *Salmonella recA* mutant was also sensitive to exogenous H_2O_2 in aerated rich medium[26]. Moreover, *recG*, recombination and DNA repair gene[51], showed a stronger phenotype than *recA* mutant. The *regG* deletion in *Salmonella* caused the growth rate reduction by 52% for H_2O_2L and 60% for H_2O_2H. This disruption in *recG* also caused the cells to stay in lag phase for a longer time in

the presence of H_2O_2 as compared to LB; the lag time increased by a 62-fold and a 159-fold for H_2O_2L and H_2O_2H, respectively. In the blood of patients with *Salmonella* Typhi bacteremia, the proteins encoded by *recA*, *recG*, and *xthA* genes were detected, suggesting these proteins are actively expressed in the blood environment[52]. The XthA protein is another enzyme that participates in DNA repair mechanism induced by H_2O_2 and iron-mediated Fenton reaction. The *xthA* encodes exonuclease III, which repairs the damaged DNA. We found that the *xthA* gene was required based on the Tn-seq assay and its mutant had a reduced fitness score of -3.06 for H_2O_2L and a -4.38 for the H_2O_2H. Further, *Salmonella* lacking the *xthA* increased the lag time by 8-fold and a 12-fold for H_2O_2L and H_2O_2H, respectively. Targeted-proteomics showed upregulation of XthA (1.64-fold) in response to H_2O_2. *Salmonella* enterica serovar Enteritidis defective in *xthA* is susceptible to egg albumin[53]. *E. coli xthA* mutant is hypersensitive to H_2O_2[54]. The *xthA* is also required for *Mycobacterium tuberculosis* to infect C57BL/6J mice[55]. In addition to the aforementioned genes involved in DNA repair system, *uvrA* encoding Holliday junction DNA helicase motor protein, *uvrC* encoding exonuclease ABC subunit A, *uvrD* encoding DNA-dependent helicase II, and *polA* encoding DNA polymerase I were among top of the list of the genes identified by Tn-seq as required for resistance to H_2O_2. Collectively, DNA repair system is crucial for the survival of the *Salmonella* in a niche that contains H_2O_2.

2.3.12 Flagellar genes have a role for H_2O_2 resistance

Some flagellar genes, *fliC* and *fliB*, were shown to be important for resistance to H_2O_2. These two genes were identified by Tn-seq and their proteins were shown to be upregulated. *Salmonella* lacking either of these genes exhibited a strong phenotype in the presence of H_2O_2. During 24 h of incubation, *fliC* and *fliB* mutants could not grow in both H_2O_2 conditions. However, growth of *fliD* mutant was not affected by H_2O_2. The *fliC* was shown to have a role in *Salmonella* Typhi interaction with human macrophages and *Salmonella* Typhimurium *fliB*

mutant was defective in swarming motility[56, 57]. Currently it remains unclear how flagella genes can be involved in the resistance of *Salmonella* to oxidative stress, which warrants future study into this direction.

2.3.13 Fe-S cluster biogenesis system is required for H_2O_2 resistance

Salmonella requires the genes from Fe-S cluster biogenesis system in order to resist H_2O_2. Our Tn-seq analysis identified 5 genes in this system that are required for the resistance. In *isc* operon (Fe-S cluster), *iscA*, *hscB*, and *hscA* were among the genes required to resist H_2O_2. Particularly, the *hscA* is on the top of the gene list identified by Tn-seq. In *E. coli*, this operon is regulated by *iscR*, iron sulfur cluster regulator[58]; in *Salmonella* the gene *iscR* encoding this transcription regulator is named *yfhP*. The HscB and HscA chaperones are believed to be involved in the maturation of Fe–S proteins[59, 60]. The second operon that is involved in Fe-S protein biogenesis is the *suf*, sulfur mobilization operon. Tn-seq found that two genes in this operon were required for *Salmonella* to resist H_2O_2; *sufS* and *sufC*. *Salmonella* mutant lacking *sufS* exhibited a strong phenotype in the presence of H_2O_2 and could not grow during the 24 h of incubation as compared to LB. The SufS with SufE in *E. coli* form a heterodimeric cysteine desulphurase and SufB, SufC, and SufD form a pseudo-ABC-transporter that could act as a scaffold[60]; this operon is regulated by OxyR[14]. The other known genes in these two operons that are present in *Salmonella* are *iscA*, *sufA*, *sufB*, and *sufD*; they showed a reduced fitness, while their p values were > 0.05. The damage of Fe-S clusters is not only problem for the defective proteins, but also it fuels the Fenton reaction via the released iron and H_2O_2[10]. Thus, *Salmonella* uses Fe-S cluster repair system as an arsenal to overcome the damage imposed by H_2O_2.

2.3.14 DNA adenine methylase is important for H_2O_2 resistance

DNA adenine methylase genes, *dam* and *damX*, are involved in *Salmonella* resistance

against H_2O_2. Our Tn-seq data showed that fitness of *dam* and *damX* mutants were reduced in the presence of H_2O_2. To confirm this, *Salmonella dam* mutant was grown in both conditions. Under H_2O_2L, the growth rate was reduced by 42% as compared to the mutant in LB and the mutant could not grow under H_2O_2H during the 24 h of incubation. In addition, the lag time of the *Salmonella dam* mutant was extended by a 19-fold for H_2O_2L. While the *Salmonella damX* mutant displayed a moderate phenotype as compared to the *dam* mutant, the *damX* mutant also showed that the growth rate decreased by 23% and 33% for H_2O_2L and H_2O_2H, respectively. The lag time was extended for this mutant by a 25-fold and a 49-fold for H_2O_2L and H_2O_2H, respectively. The *dam* regulates virulence gene expression in *S*. Typhimurium[61]. The different levels of Dam affects virulence gene expression, motility, flagellar synthesis, and bile resistance in the pathogenic *S*. Typhimurium 14028S[62]. Dam methylation activates the gene that are involved in lipopolysaccharide synthesis[63]. Moreover, *Salmonella* defective in *damX* is very sensitive to bile[64]. Collectively, our study demonstrates the critical role of DNA adenine methylase in *Salmonella* resistance against H_2O_2.

2.3.15 Other genes for H_2O_2 resistance

Beside the important pathways described above, there were many additional genes also important for resistance to H_2O_2. Among those, the 3 unrelated genes, *rpoS*, *pgm*, and *tonB*, are important ones that deserve more attention. The *rpoS* mutant showed reduced fitness and its protein was upregulated in the presence of H_2O_2. *Salmonella* mutant defective in *rpoS* showed a strong phenotype when grown in the presence of H_2O_2. The *rpoS* encodes the alternative sigma factor σ^S, subunit of RNA polymerase; it is the master regulator of stress response[65]. This implies that *rpoS* is an important component of the genetic regulatory network that *Salmonella* employs in order to resist H_2O_2. Furthermore, the fitness of *pgm* mutant was reduced and its protein was upregulated in the presence of H_2O_2. Knock out of *pgm* in *Salmonella* caused a decrease in growth rate, increase the lag phase time, and reduce the

maximum OD_{600} in the presence of H_2O_2. The *pgm* encodes phosphoglucomutase which required for catalysis of the interconversion of glucose 1-phosphate and glucose 6-phosphate[66]. This gene contributes to resistance against antimicrobial peptides, is required for *in vivo* fitness in the mouse model, and participates in LPS biosynthesis[67]. Lastly, the fitness of *tonB* mutant was also reduced. *Salmonella* lacking *tonB* exhibited a strong phenotype in the presence of H_2O_2 as compared to the mutant grown in LB. The gene mediates iron uptake in the *Salmonella*[45]. In addition, seven of the genes identified in our study (*proC*, *arcA*, *barA*, *exbD*, *flhD*, *fliC*, and *fliD*) were previously shown to be important for interaction of *Salmonella* Typhi with human macrophages[56].

2.4 Conclusion

In summary, we applied Tn-seq and proteomic analysis to find the genes and proteins that are required in *S.* Typhimurium to resist H_2O_2 *in vitro*. As the concentration of H_2O_2 increased, the growth rate reduced, the lag time extended, the fitness of mutants decreased, and some proteins were differentially expressed. Validation of Tn-seq results with individual mutant assays indicated the accuracy of the identified genes in response to the two H_2O_2 concentrations. The targeted-proteomics had a good agreement with Tn-seq. We found many genes that have not been associated to resistance to H_2O_2 previously and these genes will be focus of our future research. *Salmonella* employs multiple pathways to resist H_2O_2 and the most important ones are ROS detoxifying enzymes, amino acid biosynthesis (*aroK* and *aroB*), putative iron transporters (*ybbK*, *ybbL*, *ybbM*), iron homeostasis, Fe-S cluster repair, DNA repair, flagellar and DNA adenine methylase genes. The genes identified in this study will broaden our understanding on the mechanisms used by *Salmonella* to survive and persist against ROS in macrophages.

Our unbiased system-wide approach, Tn-seq, was successful in identifying novel

genetic determinants that have not been implicated previously in *Salmonella* resistance to oxidative stress. Furthermore, the combined use of quantitative proteomic approach has provided additional insights on the function or mode of action of the identified genetic determinants in resisting oxidative stress. As expected, the majority of the proteins important for resistance to H_2O_2 were upregulated in response to the same stressor. However, the expression level did not increase for some proteins, in spite of their known roles in resistance to H_2O_2. Interestingly, the downregulation of Dps and other proteins was counterintuitive to the common mode of protein regulation and function, yet it may point to some unknown aspects of how *Salmonella* regulates the expression of those proteins to better cope with the oxidative stress during infection in macrophage. The genes identified in this study will broaden our understanding on the mechanisms used by *Salmonella* to survive and persist against ROS in macrophages.

2.5 Methods

2.5.1 Construction of Tn5 mutant library

We mutagenized *Salmonella* enterica subsp. enterica serovar Typhimurium str. ATCC 14028S (with spontaneous mutation conferring resistance to nalidixic acid (NA)), by biparental mating using *Escherichia coli* SM10 λ*pir* carrying a transposon-delivery plasmid vector pBAM1 (Ampicillin (Amp) resistance) as a donor strain[68]. The plasmid pBAM1 was generously provided by Victor de Lorenzo (Systems and Synthetic Biology Program, Centro Nacional de Biotecnología, Madrid, Spain). The donor strain*, E. coli* Sm10 λpir (pBAM1), was grown overnight in LB with 50 μg/ml Amp and recipient strain was grown in LB with 50 μg/ml NA at 37°C. Equal volumes (1 ml) of donor and recipient were mixed, centrifuged, washed in 10 mM $MgSO_4$, and re-suspended in 2 ml PBS (pH 7.4). Then, the mating mixture was concentrated and laid on a 0.45-μm nitrocellulose filters (Millipore). The filter was incubated at 37°C on the surface of LB agar plate. After 5 h of conjugation, the cells on the filter was

washed in 10 mM $MgSO_4$, and resuspended in 1 ml MgSO4. The conjugation mixture was plated on LB agar containing 50 μg/ml NA and 50 μg/ml kanamycin (Km). After approximately 24 h at 37°C, we scraped the colonies into LB broth containing 50 μg/ml Km and 7% DMSO. The yield was approximately 68,000 individual colonies from each conjugation. Five independent conjugations were conducted to yield approximately 325,000 mutants. The library was stored at −80°C in aliquots. To determine the frequency of the mutants that have been produced by integration of the entire delivery plasmid, the colonies were picked randomly and streaked on LB plates (Km) and LB plates (Km and Amp). It was shown that ~20% of the cells in the library were resistant to Amp, indicating a significant portion of the Km-resistant colonies was not from authentic transposition events.

2.5.2 Measuring growth responses of *S.* Typhimurium to H_2O_2

To determine the effect of H_2O_2 concentrations on growth parameters, overnight culture of the wild type *S.* Typhimurium 14028s was inoculated into fresh LB broth media (1:200 dilution) to give a seeding concentration corresponding to OD_{600} of ~0.1. The LB broth contained freshly prepared H_2O_2 to give the final concentrations ranging from 0.05 to 10 mM. The cultures were directly added into 96-well microplate (200 μl/well). The microplate was incubated in a Tecan Infinite 200 microplate reader at 37°C, with shaking duration 5 s, shaking amplitude 1.5 mm, and reading OD_{600} every 10 min. The number of replicates were at least three. The lag time, growth rate, and maximum OD_{600} were calculated using GrowthRates script[69]. Growth Rate % decrease was calculated as follows[70]: Growth Rate % decrease = $((\mu_{PC} - \mu_S)/\mu_{PC}) \times 100$; where μ = the maximum slope (growth rate), μ_{PC} = growth rate of positive control (without H_2O_2), μ_S = growth rate in the presence of H_2O_2.

2.5.3 Selection of the mutant library for Tn-seq analysis

The transposon library was thawed at room temperature and diluted 10^{-1} in fresh LB

broth. To activate the library, the diluted library was incubated at 37°C with shaking at 225 rpm for an hour. Then, the culture was washed twice with PBS and resuspended in LB broth medium. The library was inoculated to 20 ml LB broth and LB broth supplemented with either 2.5 or 3.5 mM H_2O_2 (H_2O_2L and H_2O_2H, respectively), seeding CFU was 3.5 x 10^6 per ml. Then, when the cultures reached mid-exponential phase, OD_{600} of 2.7 (~1.17 x 10^8 CFU/ml), the incubation was stopped, and the culture was immediately harvested by centrifugation, and stored at -20°C.

2.5.4 Preparation of Tn-seq amplicon libraries

Genomic DNA was extracted from the harvested cells using DNeasy Blood & Tissue kit (Qiagen), and quantified using Qubit dsDNA RB Assay kit (Invitrogen). As described above, 20% of the mutants in the library were the result of the integration of pBAM1 into chromosome. To remove the Tn5-junction sequences originated from the plasmid in the Tn-seq amplicon libraries, genomic DNA was digested with PvuII-HF (New England Biolabs), which digests immediately outside the inverted repeats on both sides of Tn5 in pBAM1, and purified with DNA Clean & Concentrator-5 kit (Zymo Reaerch). Then, a linear PCR extension was performed using a Tn5-specific primer in order to produce single stranded DNA corresponding to Tn5-junction sequences. To increase the specificity in extending into Tn5-junction sequences, the linear PCR was conducted with a dual priming oligonucleotide Tn5-DPO (5'-AAGCTTGCATGCCTGCAGGTIIIIICTAGAGGATC-3') that is specific to Tn5 end[71]. The PCR reaction contained 25 µl Go Taq Colorless Master Mix (Promega), 20 µM Tn5-DPO primer, 100 ng gDNA, and 50 µl MQ-H_2O. The PCR cycle consisted of the initial denaturation at 95°C for 2 min, followed by 50 cycles at 95°C for 30 sec, 62°C for 45 sec, and 72°C for 10 sec. The PCR product was purified with DNA Clean & Concentrator-5 kit and eluted in 13 µl TE buffer. After that, C-tail was added to the 3' end of the single-stranded DNA. The C-tailing reaction was consisted of 2 µl terminal transferase (TdT) buffer (New England

Biolabs), 2 µl $CoCl_2$, 2.4 µl 10 mM dCTP, 1 µl 1 mM ddCTP, 0.5 µl TdT and 13 µl purified linear PCR product. The reaction was performed at 37°C for 1 h and the enzyme was inactivated by incubation at 70°C for 10 min. The C-tailed product was purified with DNA Clean & Concentrator-5 kit and eluted in 12 µl TE. Next, the exponential PCR was performed with forward primer, P5-BRX-TN5-MEO, 5'-AATGATACGGCGACCACCGAGATCTACA CTCTTTCCCTACACGACGCTCTTCCGATCTNNNNAG-6 nt barcode-CCTAGGCGGCC TTAATTAAAGATGTGTATAAGAG-3' and reverse primer, P7-16G, 5'-CAAGCAGAAGA CGGCATACGAGCTCTTCCGATCTGGGGGGGGGGGGGGG-3' to attach Illumina adapter sequences along with the sample barcodes. The PCR reaction contained 25 µl Go Taq Green Master Mix, 10 µM P5-BRX-TN5-MEO primer, 10 µM P7-16G primer, 1 µl purified C-tailed genomic junctions, and MQ-H_2O to 50 µl; the PCR condition started with initial denaturation at 95°C for 2 min, followed by 36 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 20 sec, with the final extension at 72°C for 5 min. Then, the size selection of the DNA was performed using agarose gel electrophoresis. The 50 µl PCR products were incubated at 60°C for 15 min and incubated on ice for 5 min, and immediately loaded on the 1% agarose gel in 0.5% TAE buffer. After running the gel, the DNA fragment of size 325-625 bp was cut and put in a microtube for each sample. The DNA was extracted from the gel using Zymoclean Gel DNA Recovery kit (Zymo Research). The prepared DNA libraries were quantified using Qubit dsDNA RB Assay kit. Since each library has its own barcode, the libraries were combined and sequenced on a flow cell of HiSeq 3000 using single end read and 151 cycles (Illumina) at the Center for Genome Research & Biocomputing in Oregon State University.

2.5.5 Tn-seq data analysis

The preliminary data analysis was conducted by using a super computer in the High Performance Computing Center (AHPCC) at the University of Arkansas. The libraries that were multiplexed for sequencing were de-multiplexed using a custom Python script. The script

searched for the six-nucleotide barcode for each library for perfect matches. In order to extract the transposon genomic junctions, we used Tn-Seq Pre-Processor (TPP) tool[30] with some modifications in the script. The TPP searched for the 19 nucleotide inverted repeat (IR) sequence and identified five nucleotides (GACAG) at the end of the IR sequence, allowing one nucleotide mismatch. The Tn5-junctions that start immediately after GACAG were extracted and the C-tails at the end of junctions were removed. Tn5-junction sequences less than 20 nucleotides were discarded and remaining Tn5-junctions were mapped to the *Salmonella* enterica serovar Typhimurium 14028S genome and plasmid using BWA-0.7.12[72]. To identify genes that are required for H_2O_2 resistance, the following three Tn-seq analysis tools were used for comparative analysis: (i) ARTIST[28]: the genomic junctions were mapped to the reference genome using Bowtie 2.2.7[73]. The number of insertions and reads were determined for genes and intergenic regions. The data were normalized with default script in the ARTIST. Then, the relative abundance of Tn5 insertions in the output library versus the input were calculated. Later, the *p* values were calculated from a 100 independent Mann–Whitney U test (MWU) analysis that were carried out on input and output data for each gene. Finally, the genes were considered conditionally essential if the *p* values were ≤ 0.05 in the 90 of the 100 MWU tests. (ii) Tn-seq Explorer[29]: The output SAM files from the TTP were used as input to the Tn-seq Explorer. The unique insertions with less than 20 reads were removed from the input and outputs. Using the window size of 550 and excluding 5% of beginning of genes and 20% of the end of genes, Essentiality Index (EI), number of unique insertions, and total number of reads per gene were counted. The EI of more than 10 were removed from the input. Genes with less than 300 nucleotides were removed. Deferential EI were calculated from input and outputs (ΔIE = output EI–input EI) and genes with ΔIE more than -1 were removed. Log2 fold change of reads were calculated from input and output (Log2FC = log2 (output reads/input reads)) and the genes were ranked based on the Log2FC value from least value to highest. The genes with

Log2FC value of less than -2 from the H_2O_2H and present in H_2O_2L were considered conditionally essential. (iii) TRANSIT[30]: The output wig files from the TTP was used as input data file for TRANSIT. The comparative analysis was conducted with Tn5 resampling option. The reads were normalized with trimmed total reads (TTR). Insertions outside the 5% and 10% sequences from 5'- and 3'- ends were removed, respectively. The genes were considered conditionally essential if p values ≤ 0.05.

2.5.6 Phenotypic evaluation of individual deletion mutants

The mutants were obtained from *Salmonella enterica* subsp. *enterica*, 14028s (Serovar Typhimurium) Single-Gene Deletion Mutant Library through BEI Resources (www.beiresources.org). The overnight cultures of *S.* Typhimurium mutants were added into fresh LB broth media containing freshly prepared H_2O_2 (2.5, or 3.5 mM/ml) (1:200 dilution) to give seeding OD_{600} of 0.1. The cultures were directly added into 96-well microplates and incubated in Tecan Infinite 200 at 37ºC for 24 h. The lag time, growth rate, and maximum OD_{600} were calculated using GrowthRates[69].

2.5.7 Sample preparation for proteomics and mass spectrometry analysis

The overnight culture of the wild type *S.* Typhimurium 14028 was diluted 1:200 in 50 ml LB medium, and LB containing either 2.5 or 3.5 mM H_2O_2 in a 300-ml flask. The cultures were grown until mid-exponential phase (OD_{600} of 2.7), and the 50 ml volume of cultures were used for a total protein extraction by using Qproteome Bacterial Protein Prep kit (Qiagen). Disulfide bonds within proteins were reduced with 2-Mercaptoethanol and separated by SDS-PAGE gel electrophoresis. For each condition, there were three lanes with approximately 300 μg of proteins. The gel then was stained via coomassie blue dye. The gel portions of 3 lanes for each condition were cut out and chopped into small pieces, pooled together, washed twice with 50 mM NH_4HCO_3, destained with NH_4HCO_3/ 50% Acetonitrile (ACN), and dried with

pure ACN. Then, the proteins were reduced using 10 mM Dithiothreitol in 50 mM NH_4CO_3 and the alkylation was conducted with 10 mg/ml Iodoacetamide Acid in 50 mM NH_4CO_3. After that, the proteins were washed with NH_4HCO_3, and dried with pure ACN. Mass spectrometry grade Trypsin gold from Promega (~20 ng/µl in 50 mM NH_4HCO_3) was added to dried gels, and left it overnight for efficient in-gel digestion of the proteins at 37ºC. During the digestion, tryptic peptides diffused out into the solution. Gel pieces then were extracted three times by 50% CAN/0.1% TFA solution and incubated at 37^{o}C for 15 minutes. Later, these digests were analyzed by ESI-LC-MS/MS at State Wide Mass Spectrometry Facility, University of Arkansas at Fayetteville. Data dependent analysis (DDA) for the in-gel trypsin digested samples from each condition were performed by using an Agilent 1200 series micro flow HPLC in line with Bruker Amazon-SL quadrupole ion trap ESI mass spectrometer (QIT-ESI-MS). All the ESI-MS analyses were performed in a positive ion mode using Bruker captive electrospray source with a dry nitrogen gas temperature of 200°C, with nitrogen flow rate of 3 L/minute. LC-MS/MS data were acquired in the Auto MS(n) mode with optimized trapping condition for the ions at m/z 1000. MS scans were performed in the enhanced scanning mode (8100 m/z/second), while the collision-induced dissociation or the MS/MS fragmentation scans were performed automatically for top ten precursor ions with a set threshold for one minute in the UltraScan mode (32,500 m/z/second). Tryptic peptides were separated by reverse-phase high-performance liquid chromatography (RP-HPLC) using a Zorbax SB C18 column, (150 × 0.3 mm, 3.5 µm particle size, 300 Å pore size, Agilent Technologies), with a solvent flow rate of 4 µL/minute, and a gradient of 5%–38% consisting of 0.1% FA (solvent A) and ACN (solvent B) over a time period of 320 minutes. Tryptic peptides were then identified by searching MS/MS data in *S.* Typhimurium 14028S reference proteome database[24, 35] by using MASCOT database search software[34]. MS1 intensities of the integrated areas of these identified tryptic peptides were compiled and grouped in skyline software according to the

replicates/conditions to perform statistical analysis. Targeted protein work were performed using Shimadzu UPLC-20A coupled to 8050 triple quadrupole ESI-MS with heated probe. Sequence specific fragment ion intensities from at least three unique tryptic peptides from the protein of interest were used in the protein quantitation. Multiple reaction monitoring (MRM) events corresponding to sequence specific fragment ions derived from the precursor tryptic peptides were targeted to operate at a certain specific retention time intervals predicted by in house retention time library. This library was generated using the correlation of relative hydrophobicity of the tryptic peptides with their retention times (RT) from highly common housekeeping proteins, for the UPLC method used in this analysis as described below. While the RT were correlated well within 99% confidence, sufficient number of sequence specific fragment ions were used as basis for identification of the tryptic peptide by MS/MS alone. Specificity and the confidence was achieved by incorporating RT prediction. In addition to the application of skyline in quantitation, skyline software was also used in predicting RT and optimizing parameters such as collision energies and voltages with the help of Shimadzu Labsolution software. Tryptic peptides were separated by reverse-phase ultra-high-performance liquid chromatography (RP-UPLC) compatible Shimadzu C18, 1.9-micron particle size, 50x2.1 mm column (SN # 16041880T), with a solvent flow rate of 0.3 mL/minute, and a gradient of 5%–90% consisting of 0.1% FA (solvent A) and 0.1% FA in ACN (solvent B) over a time period of 10 minutes.

2.5.8 Accession numbers

Tn-seq sequencing data are available on NCBI Sequence Read Archive. LB: PRJNA352537; H_2O_2L: PRJNA352862; H_2O_2H: PRJNA352865.

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2.7 Author Contributions

Conceived and designed the experiments: YK SK. Performed the experiments, analyzed the data, wrote the manuscript: SK. Proteomics work: RL SK AQ JL. Revised the manuscript: YK SK. All authors read the final version of the manuscript.

2.8 Figures

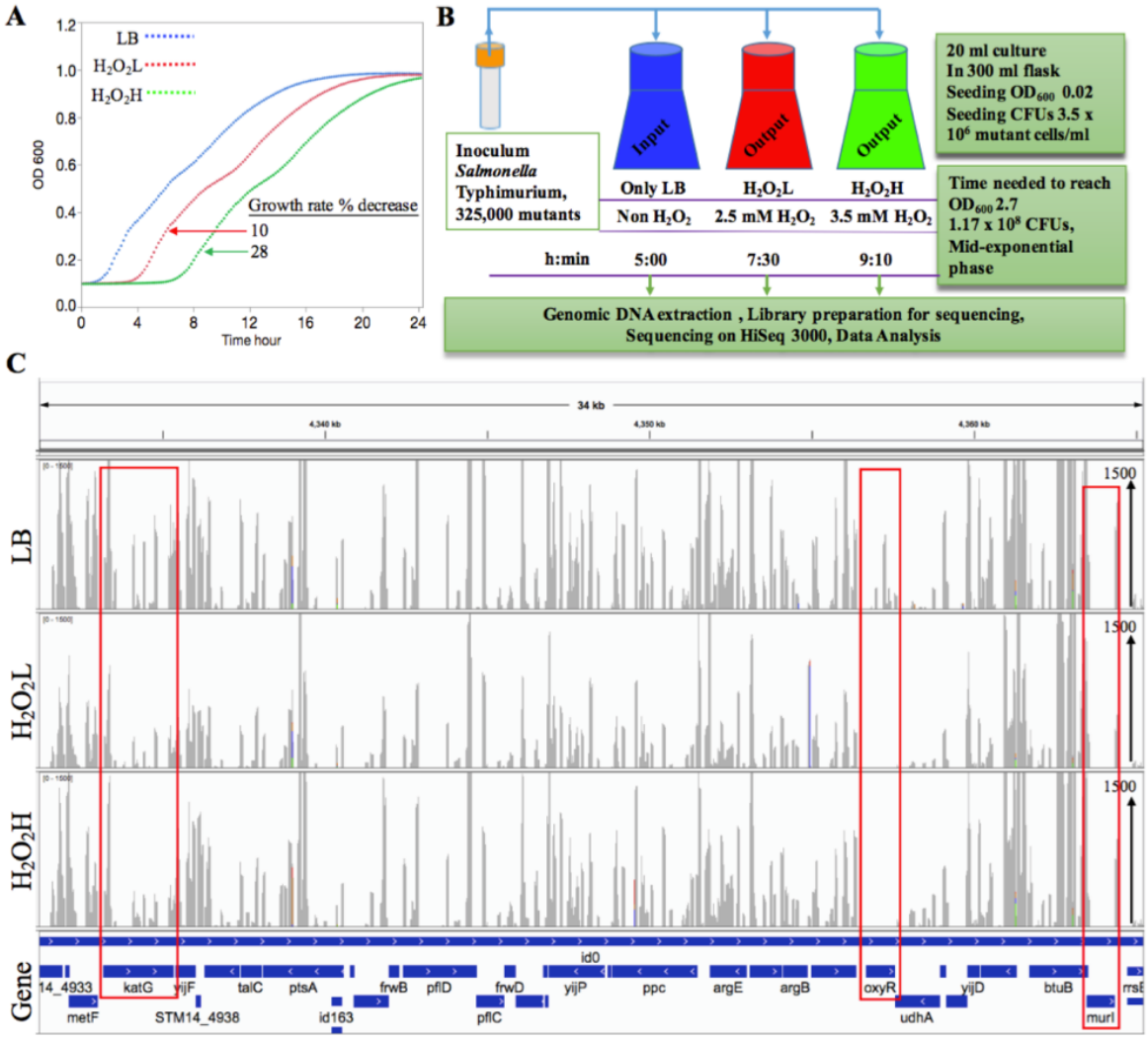

Figure 2.1. Study design and identification of the genes required for H_2O_2 resistance using Tn-seq. (A) The effect of H_2O_2 on the growth rate of wild type *Salmonella* Typhimurium. An overnight culture of bacteria was diluted 1:200 in the LB medium contains either 2.5 mM H_2O_2 (H_2O_2L), 3.5 mM H_2O_2 (H_2O_2H), or LB without H_2O_2 was used as control. The cultures were incubated at 37°C for 24 h in a 96-well plate. The reduced growth rates for the H_2O_2 were in comparison to the control. In the all growth curve figures in this work, the blue color represents LB (no H_2O_2), the red is H_2O_2L, and green is H_2O_2H. (B) Schematic representation of the Tn-seq study. The *Salmonella* transposon mutant library was inoculated into LB and LB contains H_2O_2L or H_2O_2H. The three cultures were grown until they reached mid-exponential phase. The DNA was extracted from each culture and subjected to library preparation, sequencing, and data analysis. (C) The Tn-seq profile of the three conditions. It shows 34 kb of the *Salmonella* genome which starts with *metF* gene and ends with *rrsB*, horizontal axis. The height of vertical axis represents number of reads which is 1500 sequencing reads. The highlighted genes in red are *katG*, catalase peroxidase, was tolerated insertions in the both H_2O_2 conditions; *oxyR* was not tolerated insertions in presence of H_2O_2 and indicated that the gene is required to H_2O_2 resistance; *murI*, glutamate racemase, was not tolerated any insertions at all and it was considered an essential gene, *murI* is required for the biosynthesis of a component of cell wall peptidoglycan.

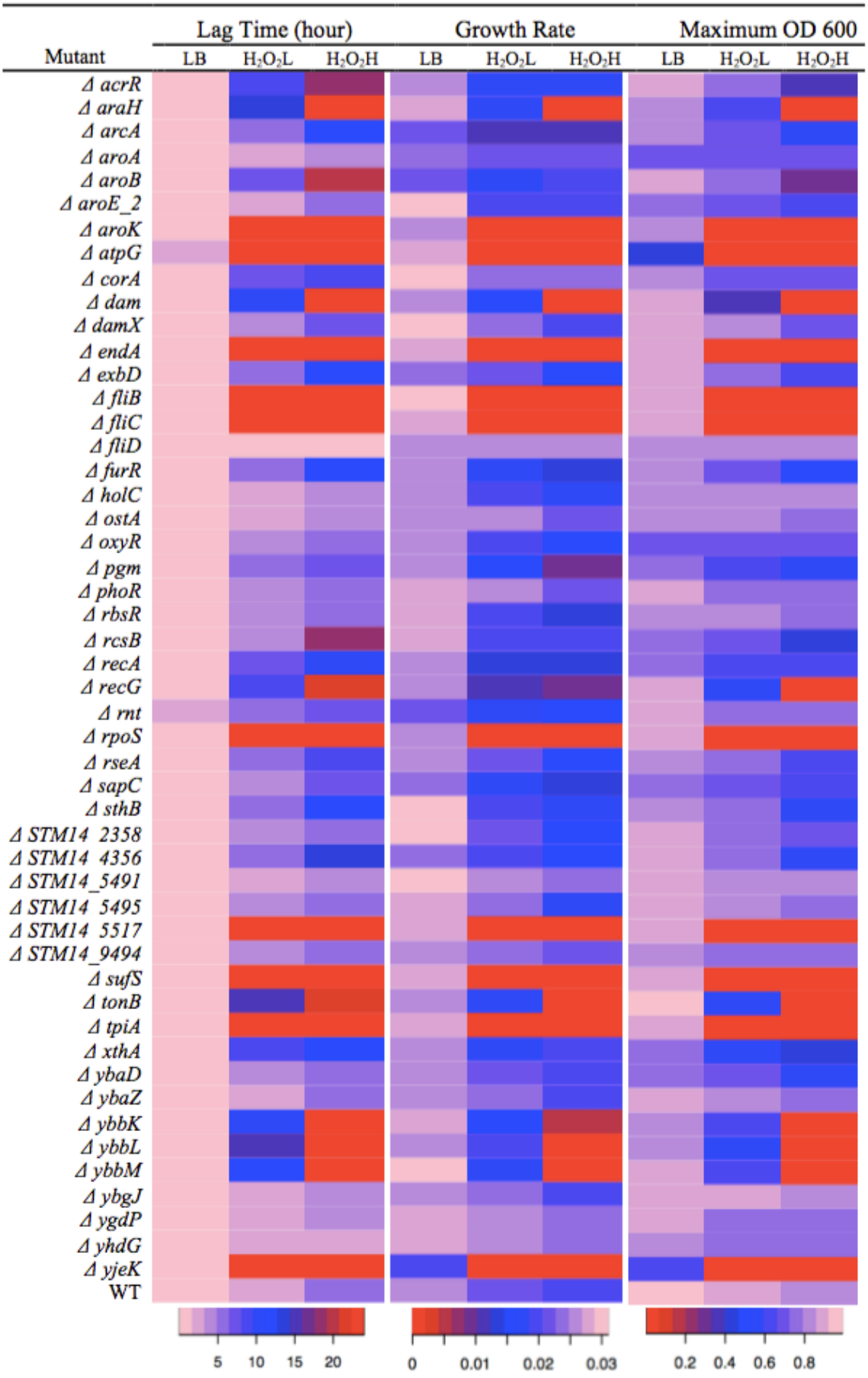
Lag Time (hour)
Growth Rate
Maximum OD 600
Mutant
LB
H2O2L
H2O2H
Δ acrR
Δ araH
Δ arcA
Δ aroA
Δ aroB
Δ aroE_2
Δ aroK
Δ atpG
Δ corA
Δ dam
Δ damX
Δ endA
Δ exbD
Δ fliB
Δ fliC
Δ fliD
Δ furR
Δ holC
Δ ostA
Δ oxyR
Δ pgm
Δ phoR
Δ rbsR
Δ rcsB
Δ recA
Δ recG
Δ rnt
Δ rpoS
Δ rseA
Δ sapC
Δ sthB
Δ STM14_2358
Δ STM14_4356
Δ STM14_5491
Δ STM14_5495
Δ STM14_5517
Δ STM14_9494
Δ sufS
Δ tonB
Δ tpiA
Δ xthA
Δ ybaD
Δ ybaZ
Δ ybbK
Δ ybbL
Δ ybbM
Δ ybgJ
Δ ygdP
Δ yhdG
Δ yjeK
WT
5 10 15 20
0 0.01 0.02 0.03
0.2 0.4 0.6 0.8

Figure 2.2. Growth curve of 50 mutants and a wild type *Salmonella* in LB, H_2O_2L and H_2O_2H. The lag phase time, growth rate, and maximum OD_{600} of the individual *Salmonella* Typhimurium mutants and the wild type in the growth conditions of LB (no H_2O_2), H_2O_2L and H_2O_2H. The overnight cultures of the mutants and wild type *Salmonella* were diluted 1:200 in the LB medium, and the LB contains either 2.5 mM H_2O_2 (H_2O_2L) or 3.5 mM H_2O_2 (H_2O_2H). The cultures were incubated at 37°C for 24 h in a 96-well plate and the OD_{600} was recorded every 10 min. The lag phase time, growth rate, and maximum OD_{600} were calculated and shown here as a graphical representation. The pale pink color indicates a short lag phase time, a high growth rate, and a high OD_{600}. The red color indicates that the bacteria was stayed in a lag phase, growth rate was close to a zero, and the OD_{600} of the culture was not raised in the 24 h time of assays. The data of this figure can be found in Table S2.4.

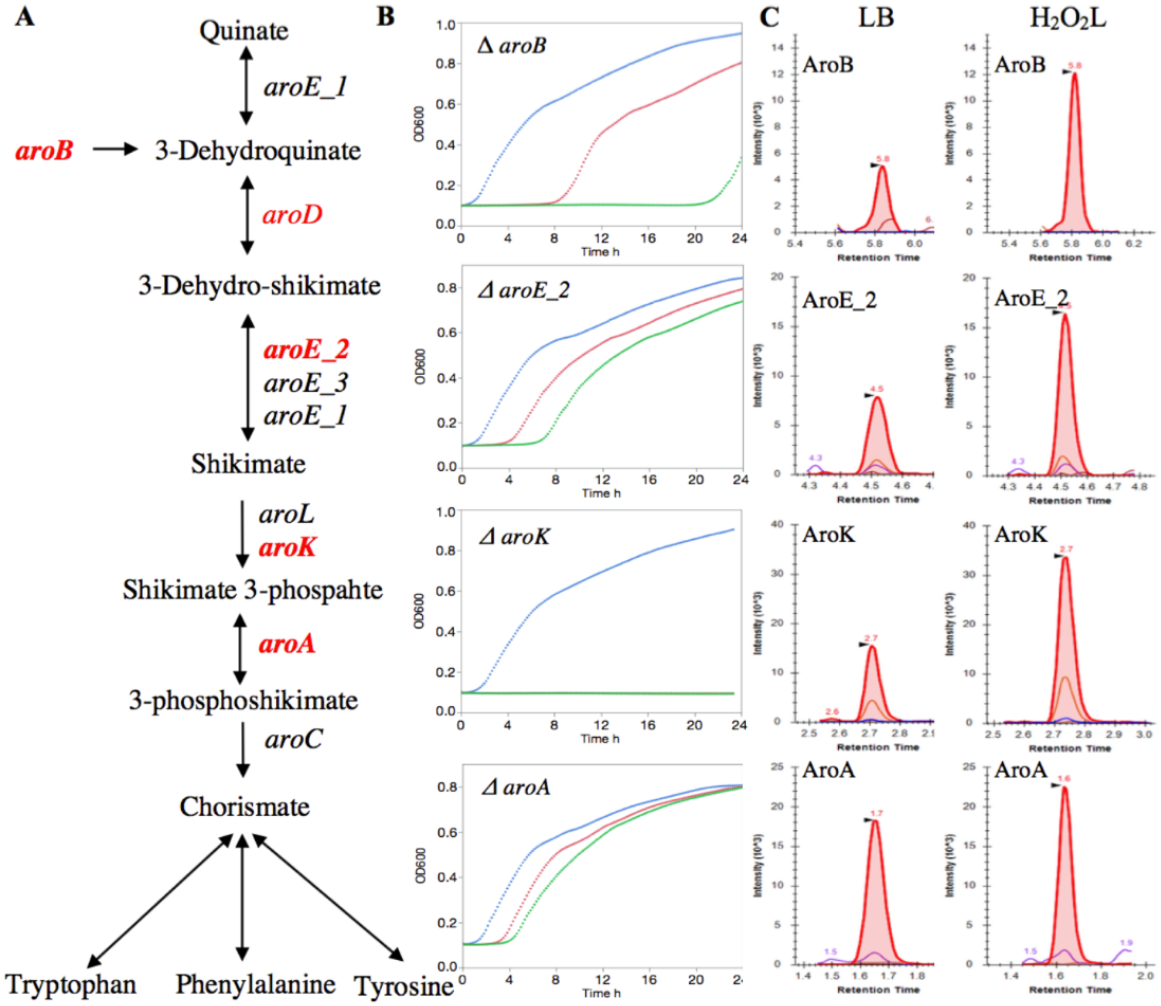

Figure 2.3. The role of aromatic amino acid biosynthesis genes in resistance to the H_2O_2. (A) Schematic representation of aromatic amino acid biosynthesis, adapted from the KEGG pathway database. The genes in red color were identified by the Tn-seq for H_2O_2 resistance in *Salmonella*. The red bold color genes were identified by the Tn-seq and the phenotypes were validated by the individual mutant assays. (B) The overnight cultures of the individual mutants were diluted 1:200 in the LB (no H_2O_2) and the LB contains either 2.5 mM H_2O_2 (H_2O_2L) or 3.5 mM H_2O_2 (H_2O_2H). The cultures were incubated at 37°C for 24 h in a 96-well plate. The colors of growth curve figures are blue for LB, red for H_2O_2L, and green for H_2O_2H. In the *ΔaroK* growth curve, the red color is under the green color. (C) Differential expression of *Salmonella* proteins in response to the H_2O_2L compared to the LB. Wild type *Salmonella* was grown in LB, H_2O_2L, and H_2O_2H until mid-exponential phase. Targeted-proteomics was quantified AroB, AroE_2, AroK, and AroA protein expressions in response to H_2O_2L. The shown peaks represent a unique peptide of the three peptides that were used of protein expression analysis.

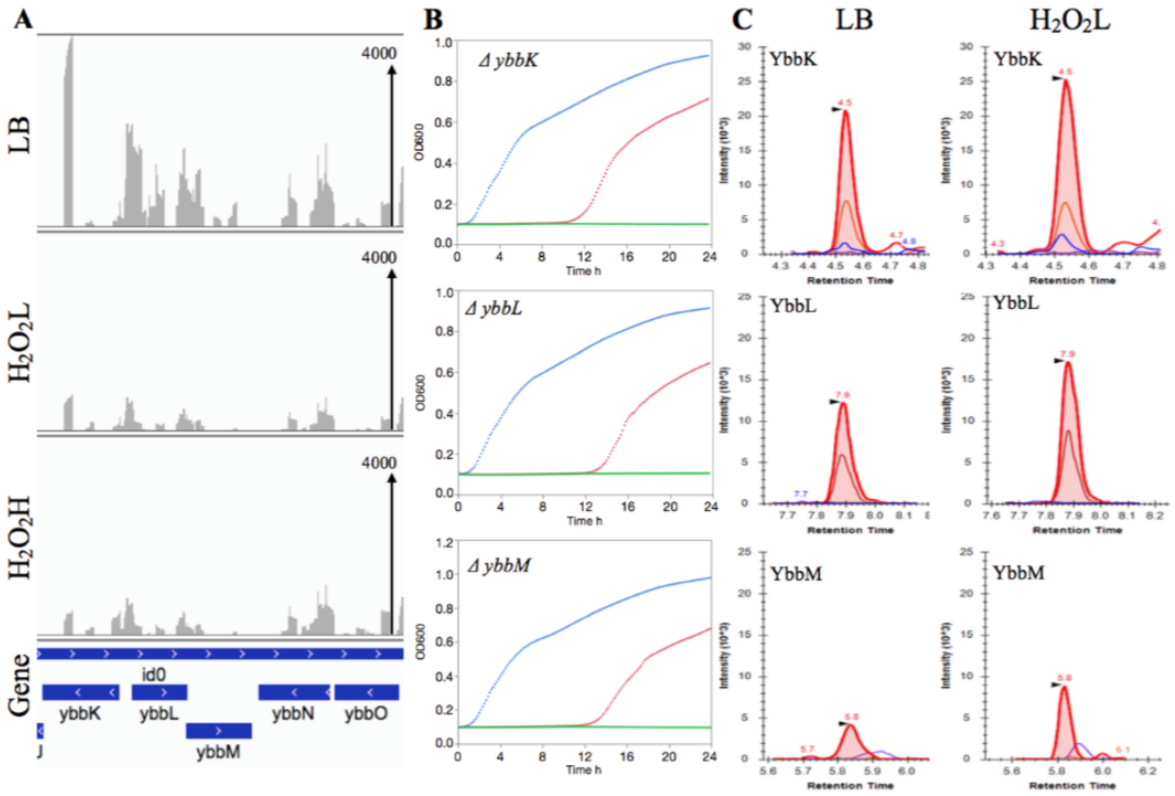

Figure 2.4. The *ybbK*, *ybbL*, and *ybbM* have a role in resistance to H_2O_2. (A) The Tn-seq profile of the LB (no H_2O_2), H_2O_2L (2.5 mM), and H_2O_2H (3.5 mM). It shows ~6 kb of *Salmonella* Typhimurium genome starts with *ybbK* and ends with *ybbO*, horizontal axis. The read scale for the conditions are 4000, vertical axis. (B) The growth curve of *ΔyybK*, *ΔyybL*, and *ΔyybM*. The overnight cultures of these three mutants were diluted 1:200 in LB, H_2O_2L, and H_2O_2H. The cultures were incubated at 37°C for 24 h in a 96-well plate. The growth curve colors are blue which represents LB, red is H_2O_2L, and green is H_2O_2H. (C) Wild type *Salmonella* was grown in LB, H_2O_2L, and H_2O_2H until mid-exponential phase. Targeted-proteomics was quantified YbbK, YbbL, and YbbM protein expressions in response to H_2O_2L. The shown peaks represent a unique peptide of the three peptides that were used of protein expression analysis.

2.9 Supplementary information

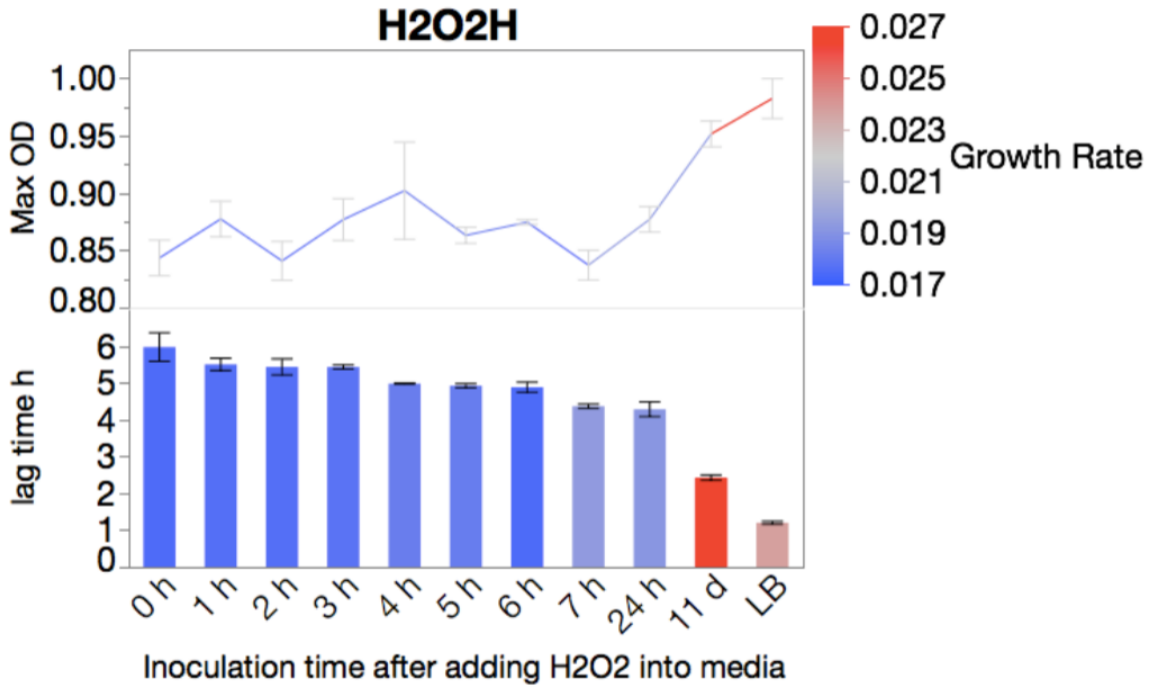

Figure S2.1. Stability of H_2O_2 in LB medium during the experiments. LB broth media supplemented with freshly diluted 3.5 mM H_2O_2 at each of indicated time points. At 0 h, immediately after adding H_2O_2 to LB broth, the media inoculated with *Salmonella* Typhimurium. At 24 h, 24 hours before the inoculation media with bacteria H_2O_2 added to media, and at 11 d, 11 days before the inoculation media with bacteria H_2O_2 added. The media supplemented with H_2O_2 left at room temperature. LB was free of H_2O_2. The cultures were incubated at 37°C for 24 h in a 96-well plate with OD_{600} reading every 10 minutes.

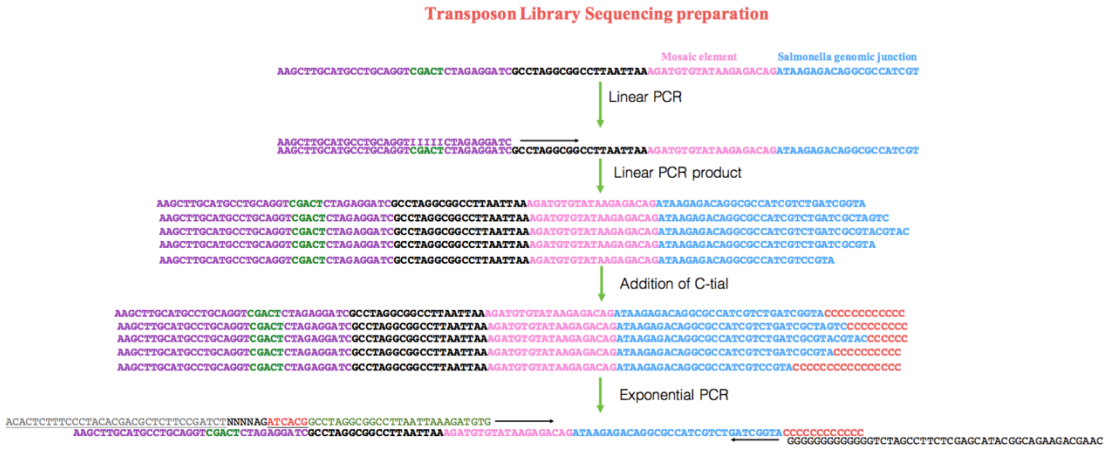

Figure S2.2. Tn-seq library preparation diagram for Illumina sequencing. The genomic DNA extracted from the selected library and subjected to two PCR amplifications. First PCR was linear and specific forward primer used to capture and amplify Tn5 junctions. Second PCR was exponential and Illumina adaptors with a barcode added. The PCR product gel purified and sequenced on an Illumina platform.

	Number of extracted GACAG_reads >20bp	Number of mapped reads	Number of unique insertions	Mean genomic length bp	Mean number of reads	Hits per nucleotides
LB	31,728,005	25,223,444	125,449	92.9	201	39
H2O2L	17,986,240	14,855,156	118,169	93.3	126	41
H2O2H	22,665,911	18,622,648	119,801	94.6	155	41
Total	72,380,156	58,701,248	363,419			
Average	20,326,075.5	16,738,902		93.95		40
STD						1.25

Table S2.1. *Salmonella* Typhimurium Tn-seq sequencing in numbers. Number of extracted reads, mapped reads, and unique insertions for LB (H_2O_2 free), H_2O_2L (2.5 mM), and H_2O_2H (3.5 mM) presented. Mean length of mapped genomic junctions and hits per nucleotide shown.

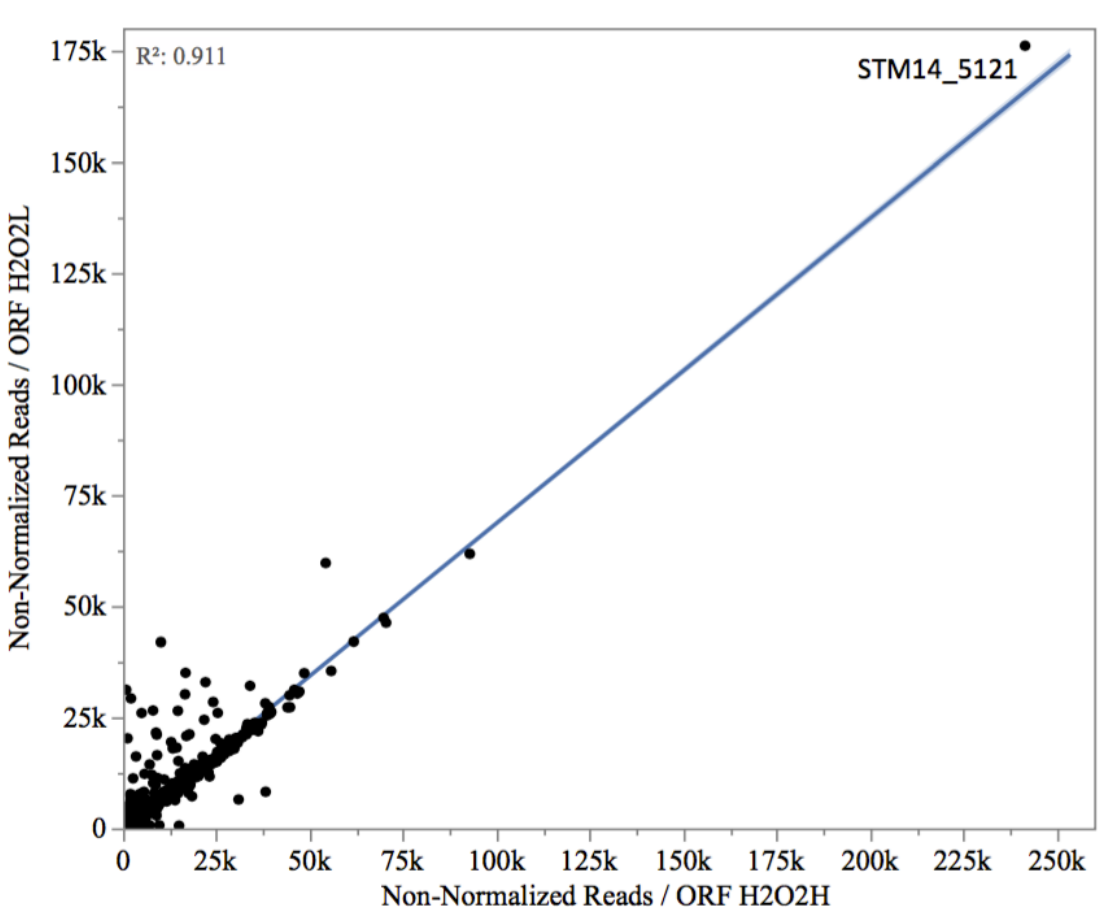

Figure S2.3. The reproducibility of Tn-seq. Correlation between reads per ORFs of *Salmonella* Typhimurium Tn-seq conditions, H_2O_2L (2.5 mM) and H_2O_2H (3.5 mM). Two ORFs excluded in this correlation, STM14_2422 and STM14_2428.

Functional Categories	Count	%	Genes
dna repair	10	7.8125	*ruvB, uvrC, ruvA, uvrD, uvrA, xthA, recR, ruvC, recA, polA*
DNA damage	10	7.8125	*ruvB, uvrC, ruvA, uvrD, uvrA, xthA, recR, ruvC, recA, polA*
cytoplasm	22	17.1875	*gidA, tpiA, uvrC, crr, uvrA, xerC, xerD, cmk, recA, gmhA, pnp, dps, argR, sufS, aroK, efP, fadR, aroB, dnaJ, aroA, yaiB, flhD*
dna recombination	7	5.46875	*ruvB, ruvA, xerC, xerD, recR, ruvC, recA*
sos response	6	4.6875	*ruvB, uvrC, ruvA, uvrD, uvrA, recA*
atp-binding	20	15.625	*atpA, sufC, ruvB, lon, uvrD, ruvA, uvrA, cmk, yjeA, recA, barA, fepC, recG, hscA, ybbL, aroK, arcB, phoL, phoR*
hydrolase	21	16.40625	*atpA, dcd, lon, ruvB, uvrD, ruvA, recC, hutG, rnt, xthA, polA, arcA, recG, apaH, yejM, endA, lepB, degS, ruvC, ygdP, spoT*
nucleotide-binding	19	14.84375	*atpA, sufC, lon, ruvB, uvrD, ruvA, uvrA, cmk, yjeA, recA, barA, fepC, recG, hscA, ybbL, aroK, arcB, phoR*
dna-binding	23	17.96875	*rcsA, rpoE, oxyR, lon, uvrD, ruvA, uvrA, xerC, xerD, yjeB, recA, acrR, arcA, polA, dps, argR, rbsR, rpoS, fadR, rcsB, fruR, priB, flhD*
metal-binding	16	12.5	*entD, iscA, uvrA, icdA, hutG, xthA, yjeB, dps, aroK, dksA, ruvC, recR, dnaJ, yhgI, pgm*
aromatic amino acid biosynthesis	5	3.90625	*aroK, aroE, aroD, aroB, aroA*
stress response	6	4.6875	*rpoE, lon, dnaJ, hfq, yaiB*
signal	14	10.9375	*yejE, fepD, barA, yejM, endA, mrdA, arcB, ybbM, degS, phoR, sthB, cysP, ompS, cbiM*
amino-acid biosynthesis	7	5.46875	*argR, aroK, aroA, aroD, aroB, aroA, proC*
DNA binding	4	3.125	*rcsA, argR, rpoS, fruR*
zinc-finger	4	3.125	*uvrA, dksA, recR, dnaJ*
transferase	20	15.625	*ybaZ, fliB, rfaF, entD, crr, slrB, cmk, barA, polA, pnp, otsA, sufS, aroK, arcC, holD, holC, arcB, dam, phoR, aroA*
dna replication	5	3.90625	*uvrD, dam, pola, dnaj, prib*
activator	5	3.90625	*rcsA, fadR, rcsB, fruR, flhD*
exonuclease	4	3.125	*rnt, recC, xthA, polA*
transport	14	10.9375	*atpA, entD, crr, yejE, fepD, corA, tonB, atpG, sapC, fliJ, exbD, sthB, cysP, ompS*
protein transport	4	3.125	*sapC, fliJ, exbD, tonB*
nuclease	4	3.125	*rnt, xthA, ruvC, polA*
magnesium	6	4.6875	*aroK, entD, xthA, corA, ruvC, pgm*
flagellum	4	3.125	*fliJ, fliD, fliC, flhD*
repressor	4	3.125	*argR, fadR, yjeB, fruR*
phosphoprotein	5	3.90625	*arcB, crr, rcsB, phoA, barA*
helicase	4	3.125	*ruvB, ruvA, uvrD, recG*
nucleotidyltransferase	4	3.125	*holD, holC, polA, pnp*
ion transport	5	3.90625	*atpaA, entD, corA, ompS, atpG*
iron	6	4.6875	*dps, entD, iscA, yjeB, yhgI*
Isomerase	6	4.6875	*slpA, rpe, tpiA, gmhA, pgm*
excision nuclease	2	1.5625	*uvrC, uvrA*
capsule biogenesis/ degradation	2	1.5625	*rcsA, rcsB*
transcription regulation	13	10.15625	*rcsA, oxyR, rpoE, yjeB, acrR, arcA, argR, rbsR, rpoS, fadR, rcsB, fruR, flhD*
Transcription	13	10.15625	*rcsA, oxyR, rpoE, yjeB, acrR, arcA, argR, rbsR, rpoS, fadR, rcsB, fruR, flhD*
dna integration	2	1.5625	*xerC, xerD*
Chromosome partition	2	1.5625	*xerC, xerD*
Chaperone	4	3.125	*sthB, dnaJ, hscB, hscA*
kinase	7	5.46875	*aroK, arcC, arcB, crr, cmk, phoR, barA*
trna processing	3	2.34375	*gidA, rnt, yhdG*
cell membrane	12	9.375	*atpA, rpe, yejE, lepB, sapC, fliJ, fepD, corA, tonB, ompS, yejM, atpG*
membrane	13	10.15625	*atpA, rpe, yejE, lepB, sapC, fliJ, fepD, corA, sthB, tonB, ompS, yejM, atpG*
dna excision	2	1.5625	*uvrC, uvrA*
nadp	3	2.34375	*icdA, aroA, proC*
cf(1)	2	1.5625	*atpA, atpG*
Protease	4	3.125	*lon, degS, lepB, araH*
cell inner membrane	7	5.46875	*atpA, lepA, sapC, corA, tonB, yejM, atpG*
two-component regulatory system	4	3.125	*arcB, rcsB, phoA, barA*
Sigma factor	2	1.5625	*rpoE, rpoS*
rna-binding	3	2.34375	*hfq, pnp*
bacterial flagellum biogenesis	2	1.5625	*fliJ, flhD*
bacterial flagellum	2	1.5625	*fliD, fliC*
atp synthesis	2	1.5625	*atpA, atpG*
Hydrogen ion transport	2	1.5625	*atpA, atpG*
Unknown ORF	15		*STM14_0196, STM14_1174, STM14_1758, STM14_1944, STM14_2358, STM14_2430, STM14_3007, STM14_3217, STM14_3219, STM14_3285, STM14_5452, STM14_5491, STM14_5494, STM14_5495, STM14_5517*

Table S2.3. List of functional categories required for *Salmonella* Typhimurium H_2O_2 resistance. SP_PIR_Keywords used with default options for functional categories analysis of the 137 genes that were required for H2O2 resistance in *S*. Typhimurium. The gene recognition by the analysis tool based on official gene symbols.

Pathways	Proteins
Ribosome	**RplB**, **RplC**, **RplD**, **RplE**, **RplI**, **RplJ**, **RplK**, **RplL**, **RplM**, **RplN**, **RplO**, **RplP**, **RplQ**, **RplR**, **RplT**, **RplU**, **RplV**, **RplW**, **RplX**, **RpmA**, **RpmD**, **RpmI**, **RpoA**, **RpoC**, **RpsA**, **RpsB**, **RpsC**, **RpsD**, **RpsE**, **RpsG**, **RpsH**, **RpsI**, **RpsJ**, **RpsK**, **RpsL**, **RpsM**, **RpsO**, **RpsS**, **RpsU**
Glycolysis / Gluconeogenesis	AdhP, Acs, **AceF**, **Eno**, AceE, PykF, Crr, **GpmA**, FbaB, **LpdA**, GlpX, Pgm, PfkB, **Pgk**
Pyruvate metabolism	Acs, **AceF**, AceE, PykF, **AccB**, Mdh, Pta, Ppc, LldD, **LpdA**, GloA, **PflB**
Pentose phosphate pathway	**PrsA**, TalB, FbaB, DeoB, RpiA, Eda, GlpX, Pgm, PfkB
Citrate cycle (TCA cycle)	SucB, **AceF**, AceE, AcnB, IcdA, Mdh, **LpdA**, GltA
Amino sugar and nucleotide sugar metabolism	GalK, ManA, Crr, GalT, GalE, YfbG, ManX, Pgm, NagZ
Propanoate metabolism	Acs, **AccB**, Pta, PrpE, PflB, PrpC
Fatty acid biosynthesis	**FabI**, **FabG**, **AccB**, FabF, **FabB**
Purine metabolism	**PrsA**, GuaB, PykF, **RpoC**, **Adk**, **RpoA**, Hpt, Ndk, DeoB
Galactose metabolism	GalK, GalT, GalE, Pgm, PfkB
Streptomycin biosynthesis	RfbA, Pgm, RfbD
Riboflavin metabolism	**RibH**, PhoN
Fructose and mannose metabolism	ManA, FbaB, GlpX, ManX, PfkB
Glyoxylate and dicarboxylate metabolism	AcnB, Mdh, Eda, GltA
Pyrimidine metabolism	Upp, **RpoC**, PyrC, **RpoA**, Ndk
Glycine, serine and threonine metabolism	**Asd**, GcvP, **LpdA**, GlyA
RNA degradation	GroEL, **Eno**, **Rho**
Two-component system	PhoN, ArcB, GlnB, CpxR, PhoP, ArcA, FliC
gamma-Hexachlorocyclohexane degradation	PhoN, DlhH
Fructose and mannose metabolism	ManA, GlpX, ManX, PfkB
RNA polymerase	**RpoC**, **RpoA**
Red are upregulated proteins, blue are downregulated, and **bold** are essential proteins/genes (based on the Tn-seq).	

Table S2.6. Deferentially expressed proteins of *Salmonella* Typhimurium in response to H_2O_2 and their pathways. *S.* Typhimurium strain 14028S grown in LB (H_2O_2 free), H_2O_2L (2.5 mM), H_2O_2H (3.5 mM) till mid-log phase. KEGG pathway analysis used to categorize deferentially expressed proteins ($p < 0.05$). Blue for downregulated proteins, red for upregulated proteins and bold represents essential proteins.

Electronic files

Table S2.2. Full list of *Salmonella* Typhimurium Tn-seq genome of the study. The list of 137 *Salmonella* required genes for H_2O_2 resistance. The full data set of Tn-seq genome analysis with three tools, ARTIST, Tn-Seq Explorer, and TRANSIT for H_2O_2L (2.5 mM) and H_2O_2H (3.5 mM).

Table S2.4. Lag time, growth rate, and maximum OD_{600} of *Salmonella* Typhimurium mutants. 50 mutants and wild-type of *S*. Typhimurium grown in LB (H_2O_2 free), H_2O_2L (2.5 mM), H_2O_2H (3.5 mM). The cultures were incubated at 37°C for 24 h in a 96-well plate with OD_{600} reading every 10 minutes. Lag time, growth rate, and maximum OD600 calculated for each mutant and compared to the wild-type.

Table S2.5. Full list of *Salmonella* Typhimurium proteomic analysis in response to H_2O_2. *S*. Typhimurium strain 14028S grown in LB (H_2O_2 free), H_2O_2L (2.5 mM), H_2O_2H (3.5 mM) till mid-log phase. Proteome profiles analyzed by utilizing ESI-LC-MS/MS in data-dependent acquisition (DDA) mode and LC-QQQ-ESI-MS for targeted proteomics.

CHAPTER THREE

Reactive Oxygen Species-Dependent Essential Pathways in *Salmonella* Typhimurium

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3.1 Abstract

The molecular mechanisms underlying antibiotic actions on bacterial cells are complex and remain enigmatic. Uncovering these mechanisms is urgently needed to utilize last-resort antibiotics properly and develop novel antibiotics against which development of drug resistance is inherently suppressed. Recently, oxidative stress has been implicated as one common mechanism whereby bactericidal antibiotics kill bacteria. Here, we expand this model to a broader range of essential pathways far beyond the targets of currently used bactericidal antibiotics. This is based on our high-resolution Tn-seq experiment in which transposon mutants with insertions in "essential genes" were rendered non-essential in. *S.* Typhimurium under iron-restricted conditions for approximately one-third of previously known essential genes. The ROS-dependent nature of these essential genes is further validated by the fact that the relative abundance of the mutants increased with more severe iron restriction. Interestingly, the targets of most antibiotics currently in use clinically, whether bacteriostatic or bactericidal, are ROS-dependent essential genes. Our observation, taken together with the previous studies, suggests that targeting "ROS-independent" essential genes may be better strategy for future antibiotic development, because under iron-restricted host condition it is more likely that (1) its antibiotic activity is not negatively influenced, and (2) development of drug resistance is reduced, due to the absence or reduced level of the ROS component in contrast to the most current antibiotics targeting "ROS-dependent" essential genes. This work expands our knowledge on the role of ROS in general essential pathways and provides novel insights for development of more effective antibiotics with reduced problem of drug resistance development.

3.2 Importance

Due to the crisis of antibiotic resistance, development of novel antibiotics that can avoid drug resistance is urgently required. Recent studies have suggested that ROS formation is a common mechanism contributing to cell death by bactericidal antibiotics. Here we showed that this model is broadly applicable to approximately one-third of all essential genes in *S.* Typhimurium, far beyond currently known targets of bactericidal antibiotics. This conclusion is supported by our genome-wide study that the transposon mutants with insertions in these "ROS-dependent" essential genes escape antibiotic action partially and can multiply under iron-restriction condition. Our finding suggests that the targeting "ROS-independent" essential genes, in contrast to most current antibiotics targeting "ROS-dependent" essential genes, may be an effective strategy to avoid weakening in antibiotic actions and development of antibiotic resistance in the iron-restricted host environment. The new insights from this study may be critical in developing novel antibiotics with reduced drug resistance.

3.3 Introduction

Essential genes are required for cell viability and growth. These genes are pivotal targets for antibacterial drugs because blocking their proteins cause cell impairment and ultimately growth inhibition or death of bacterial cells. Thus, nearly all antibiotics in clinical use target these essential pathways. However, for many natural antibiotics, the molecular targets remain unknown (1) and even if the target is known in case of bactericidal antibiotics, the cellular events that follow in response to disruption of essential pathways leading to bacterial cell death remain puzzling.

Numerous studies have shown the role of reactive oxygen species (ROS) in cell death for eukaryotes as well as prokaryotes. In eukaryotes, apoptosis and necroptosis are associated with ROS (2, 3); ferroptosis is an iron-dependent nonapoptotic form of oxidative cell death in mammalian cancer cells; these cells die as a result of ROS accumulation and the death can be prevented via iron chelators (4). In bacteria, contribution of ROS to cell death due to bactericidal antibiotics is supported by numerous studies. Kohanski et al., (5) proposed that bactericidal antibiotics regardless of their targets, induce ROS production which consequently damages biomolecules contributing to cell death, which can be averted via iron chelators. This model asserts that upon antibiotic-target interactions, consecutive specific intracellular events induce ROS formation, specifically hydroxyl radical, via Fenton reaction, through the process that involves TCA cycle-NADH depletion and destabilization of Fe-S clusters (5, 6). Furthermore, it was also shown that ROS generation elevates in bacterial cells by the attack of competitor bacteria or P1vir phage via type VI secretion system (7). Mammalian peptidoglycan recognition protein-induced bacterial killing requires ROS and the lethality of this protein can be inhibited via an iron chelator (8). Immune cells also produce ROS to kill bacterial pathogens (9). However, despite these numerous evidences on the role ROS in bacterial cell death, it is unknown if this role of ROS can be generalized to all death process of bacterial cells, and if

not, what is the scope of cellular processes to which this role is relevant.

A pathogenic bacterium possesses a few hundred essential genes that are critical for maintaining cell viability. Empirically essential genes are defined by the genes that when inactivated lead to loss of cell viability. In *E. coli* Keio collection, single-gene deletions were made for all known open reading frames, excluding 302 genes which could not tolerate disruptions and these 302 genes were considered essential (10, 11). On the other hand, transposon insertion mutant libraries coupled with next generation sequencing (Tn-seq) is a powerful method to identify essential genes (12). Tn-seq experiment have shown that the number of essential genes are 353 in *Salmonella* Typhimurium SL326 (13); 461 in *Mycobacterium tuberculosis* H37Rv (14); and 227 in *Streptococcus pyogenes* (15). Recently, a team chemically synthesized *Mycoplasma mycoid* JCVI-syn3.0 based on 473 essential genes (16). Clustered Regularly Interspaced Short Palindromic Repeats Interference (CRISPRi) was employed for phenotypic analysis of 289 essential genes in *Bacillus subtilis* that were identified by Tn-seq and confirmed that approximately 94% the putative essential genes were genuine essential genes (17).

Nearly all studies on defining essential genomes in bacteria have been conducted using stress-free nutrient-rich media for the given bacterial species under the assumption that a minimum set of the core essential genes would be best revealed under such "optimal" growth conditions. In this study, on the contrary, we analyzed our Tn-seq data to determine and characterize essential genes in *S.* Typhimurium under the restricted conditions created by different concentrations of iron chelator 2,2`-Dipyridyl (Dip) ranging from 0 to 400 μM. Our initial effort was to identify conditionally essential genes required for fitness under iron-restriction conditions. However, we unexpectedly found that a considerable portion of the genes that are categorized as essential genes in LB media (no Dip) are rendered non-essential

under iron-restriction conditions. Furthermore, the relative abundance of the transposon mutants with insertions in those essential genes increased with the increasing severity of iron restriction. We reason that this finding has significant implications in the current crisis of antibiotic resistance and may provide valuable insights for future direction for antibiotic development. Therefore, this study will mainly focus on the analysis of the essential genes under iron-restricted condition, which we termed "ROS-dependent" essential genes, and discuss the implications of our discovery.

3.4 Results and Discussion

3.4.1 Tn-seq selection

We constructed two genome-saturating Tn5 transposon libraries (Libraries –A and –AB) in which 92.6% of all ORFs had insertions (Table S3.1). To track the relative abundance of mutants in the libraries in response to iron restriction, each library was inoculated into LB media supplemented with iron chelator 2,2`-Dipyridyl (Dip) at different final concentrations of 100 (Dip100), 150 (Dip150), 250 (Dip250), or 400 μM (Dip400) and three condition of Dip-free, iron-replete, named LB-I, LBII, and LB-III, the detail in supplementary information (Fig. S3.1, S3.2, S3.3). The cultures were grown till the bacteria reached mid-log phase. We obtained 273 million (M) sequence reads from Tn5 genomic junctions in the chromosome of *S.* Typhimurium for all conditions, and 185 M sequence reads were mapped to the genome (Table S3.2). The high number of read counts and length of mapped reads allowed us to define gene essentiality with a high precision. Our initial goal in this study was to elucidate the conditionally essential genes that are required for fitness under different levels of iron restriction using Tn-seq. We found the mutants for scores of genes (139 genes) whose fitness increased by iron restriction. This observation is contrary to the currently accepted working definition of essential genes as those that cannot tolerate disruptions. It required further detailed analysis before we could accept this interesting, yet unexpected finding. Therefore, we have

conducted a systematic analysis for essential genes and comparatively analyzed the results between iron-replete and iron-restricted conditions.

3.4.2 Essential genome of *S.* Typhimurium in iron-replete and iron-restricted niches

We used rigorous analysis algorithms for essential gene identification (Fig. S3.4). As a result, we identified 336 essential genes that are required for an aerobic growth of *S.* Typhimurium 14028S in LB broths and on LB agar plates (Table S3.3). We compared the essential genes in S. Typhimurium 14028s to those in *S.* Typhimurium SL3261 identified by TraDIS approach (13). Interestingly, out of 336 genes in our essential list, 265 (80%) orthologous genes in *S.* Typhimurium 14028s were also essential in S. Typhimurium SL3261 (Table S3.4). This is a very significant overlap considering variations in genetic backgrounds of the two strains. Further, KEGG pathway analysis recognized 306 out of 336 genes and categorized them into 23 essential pathways (Fig. S3.5).

We also analyzed the essential genes from the Tn-seq data obtained from iron-restricted conditions using the same rigorous algorithms. Surprisingly, the number of essential genes under iron-restricted conditions decreased to 215 genes, which indicated that 121 genes (36%) of the 336 essential genes are not considered essential under iron-restriction conditions (Table S3.5). The number of insertions and reads in these 121 genes significantly increased under iron-restricted conditions: the average read counts in the 121 genes were 4.3 in LB-III whereas this elevated to 68 in Dip400 (Table S3.6). This is a clear evidence that the mutants of the 121 genes not only did not die but also multiplied slowly in iron-restricted conditions. In other words, chelation of iron in the media allowed the mutants of these 121 genes to escape immediate killing and to multiply.

3.4.3 Validation of the reduced number of essential genes under iron-restricted conditions

When transposon mutants grow in liquid media, the rapidly growing mutants out

compete the slowly growing ones (18, 19). As a result, Tn-seq sequencing reads cannot be obtained for these slowly growing mutants and the genes disrupted in these mutants would be considered important for fitness under that condition. To check how significant this phenomenon in our Tn-seq and whether the sequencing read counts we obtained from Dip conditions resulted from minor competitions of mutants because the growth rate in Dip400 decreased 26.4% compared to LB (Fig. S3.3). We utilized LB-I Tn-seq data. Library was recovered on agar plates following mutagenesis and colonies had enough space on the plates in order to not compete for nutrients. Although the trend showed increase of insertions in genes in LB-I compared to broth culture, LB-II, the number of essential genes to be called non essential were slight. The average read counts in the 336 essential genes (always, excluding 5% 5' end and 10% 3' end) in LB-I and LB-II were 5.1 and 2.1, respectively, while this number was 30.6 in Dip400 (Table S3.7). Therefore, this emphasizes that increasing of insertions and read counts in many essential genes in Dip conditions are not result of reduced competitions and it is likely having a connection with iron.

We next asked whether increase of insertions and read counts in essential genes of Dip conditions were due to a bias in the Tn-seq approach. We conducted analysis for identification of essential genes without data normalization and there were differences in read counts in Tn-seq conditions (Table S3.2). For instance, the total read counts in ORFs of LB-III was ~30 millions (M) versus ~16 M in Dip400, excluding intergenic regions. Two mutants, *STM14_2422* (*umuC*) and *STM14_2428*, consumed 8.7% of reads in LB-III and 27% in Dip400. Consequently, on average an insertion in LB-III had chance to get 227 reads while in Dip400 the chance was dropped to only 100 reads for an insertion (Table S3.8). This indicates that the bias in read counts was in favor to not see insertions and read counts in essential genes in iron-restricted conditions. Even though with this bias in read counts which partially produced by these two mutants, the read counts in the 121 genes were higher in Dip compared to LB.

This is a strong evidence that these 121 mutants, specifically 33 genes, are genuine slowly growing mutants in iron-restricted conditions.

3.4.4 Fitness of slowly growing mutants of essential genes increased

We next measured fitness of the 121 genes in Dip250 and Dip400 (outputs) and LB-III (input). Strikingly, fitness of 97 out of 121 genes (78%) were increased in Dip400, the rest of the 121 genes were either had increased fitness in Dip250-I, or Dip250-II. Further analysis indicated that number of essential genes with increased fitness in presence of Dip were 33 including *gyrA*, *gyrB*, and *ileS*, p values < 0.05 (Table 3.1). *gyrA*, *gyrB*, and *ileS* were not in the list of 121 genes, whereas fitness of their mutants increased significantly. This is another strong evidence that the genes with positive fitness in iron-restricted conditions are slowly growing mutants and the iron chelator cased their death.

3.4.5 Essential genes are not condition-specific

We next hypothesized that the essential genes are operationally defined depending on the specific growth conditions, and the essential gene set was changed under iron-restricted conditions. To test this hypothesis, we looked at Tn-seq that generated for other stress conditions such as H_2O_2 (20) and H_2O_2 coupled with Dip (unpublished), but we could not find any similar patterns that significant portion of the essential genes in LB medium are rendered non-essential under stress conditions. Lee et al (19) used Tn-seq to identify essential genes in *Pseudomonas aeruginosa* under 6 different conditions, and found that the essential genes were largely overlapped, but there were also condition-specific essential genes. However, in the study, the essential genes unique to each condition was a relatively small portion, which was not the case in our study in which 36% of genes became non-essential under iron-restricted conditions. These make it difficult to consider that the hypothesis is correct.

3.4.6 ROS-dependent and ROS-independent essential genes

We finally came to a conclusion that iron-restriction allowed the growth of the mutants of essential genes and ceased or slowed down the killing process. This hypothesis is related to the ROS-mediated common killing mechanisms of bactericidal antibiotics. Since its first proposal by Kohanski et al. (5), this hypothesis has been substantiated by numerous studies using different bacterial species and bactericidal antibiotics. Traditionally, the mechanisms of antibiotic action have been studied largely in terms of antibiotic-target interactions. However, numerous researches supporting the ROS-mediated killing mechanism and have shown that the interaction of antibiotic-target leads to production of ROS, contributing to the killing activity mediated by direct blocking of the basic pathways for living cells. We believe that similar processes occurred by disruption of genes of essential proteins with transposons. Thus, mutants of essential genes that grow slowly in iron-restricted conditions are ROS-dependent essential genes which are 121 genes (Table S3.6) and mutants of essential genes that do not grow in iron-restricted and iron-replete conditions are ROS-independent essential genes which are 215 genes (Fig. 3.1, Table S3.5).

Until now this proposed mechanism has been discussed with focus on the genes that have been exploited as targets of a limited number of bactericidal antibiotics. Interestingly, our Tn-seq data show that the majority of targets of the bactericidal antibiotics are ROS-dependent essential genes (Fig. 3.1), which implies that lethal effect of the knockout of the antibiotic target genes were reduced to varying extent by restriction of available iron in the media. Our Tn-seq data show that this ROS-mediated killing mechanism is linked to about one-third of the essential genes, far beyond a limited number of genes encoding targets of bactericidal antibiotics, thereby expanding the “common” nature of the ROS-mediated lethal pathway as a universal mechanism connected to a broad range of basic essential pathways for life. By our definition, based on Tn-seq, ROS-independent essential genes, 215 genes, are required for a

robust growth and viability, the cells die upon disruption of the genes, chelation of iron can not rescue their mutants from death, and their average read counts are 9.6 in Dip400. While ROS-dependent essential genes, 121 genes, are required for a robust growth and viability, the cells do not die directly upon disruption of the genes, chelation of iron can cease the death process of their mutants, they grow very slowly in iron-restricted conditions but not in iron-replete, and their average read counts are 67.9 in Dip400 (Fig. 3.2). Further, we show that these ROS-dependent essential genes are part of 9 essential pathways (Fig. 3.3).

3.4.7 Fitting ROS-dependent essential gene in ROS-mediated antibiotic killing model

ROS-mediated antibiotic killing model has a few components: (i) antibiotic-target interactions (disruption or blockage of an essential pathway), (ii) induce NADH oxidation via the electron transport chain which depends on TCA cycle, (iii) induce superoxide formation via the electron transport chain, (iv) superoxide damages Fe-S clusters and the released ferrous iron fuels Fenton reaction, (v) the consequence of Fenton reaction is hydroxyl radical formation which leads to damage of biomolecules and ultimately cell death. Juxtaposing our work to this model, we impaired the essential gene or pathway by disrupting the gene with Tn5 transposon, no antimicrobial interference. Regarding the role of TCA cycle in this model, Kohanski et al., (5) deleted the genes in TCA cycle that produce NADH and they found that *E. coli* lacking either *acnB* or *icdA* had increased survival following antibiotic treatment, however, other genes in TCA cycle pathway that follow *acnB* and *icdA* such as *sucB* and *mdh* did not have protective effect following antibiotic treatment because NADH already formed in the pathway by *acnB* and *icdA*. Astonishingly, we found that fitness of *acnB* and *icdA* increased in iron-restricted conditions (Table 3.2); *S.* Typhimurium lacking *acnB* or *icdA* can grow better in iron-restricted conditions, whereas the fitness of other mutants in TCA cycle did not change in iron-restricted conditions. This emphasizes the role of NADH and TCA cycle in ROS formation and bacterial cell death. Evidence for the contribution of TCA cycle in ROS-mediated killing has increased.

A dysfunctional TCA cycle in *Staphylococcus epidermidis* enhanced survival following β-lactam treatment (21). It has been shown in *Staphylococcus aureus* that bactericidal activity of gramicidin A is through depletion of NADH in TCA cycle (22). Previously, we showed that *icdA* required for *S*. Typhimurium survival under hydrogen peroxide and IcdA upregulated in this condition (20). We believe that *acnB* and *icdA* mutants can grow better in iron-restricted conditions because on the one hand NADH formation decreased and on the other had the iron chelator minimized amount of ferrous iron in the cell which led to diminishing of Fenton reaction. Collectively, the result demonstrates that when cell produces less NADH via TCA cycle and the intracellular ferrous iron is short, the cell grows better due to less ROS formation.

Fenton reaction requires ferrous iron and the evidence indicates that source of the iron is intracellular Fe-S clusters for ROS generation (5). As we assessed dynamics of conditionally essential genes that mediate *S*. Typhimurium survival in different iron-restricted conditions, we identified the genes that import iron from extracellular and genes that provide intracellular iron (Fig. 3.4). When iron restriction severity was low, at Dip100 and Dip150, a siderophore gene *fepD* (iron-enterobactin transporter membrane protein) was required to import iron. At Dip150 and Dip250, *tonB* was also required. It has been suggested that siderophore complexes depend on TonB to energize the active transport across membrane via TonB-ExbB-ExbD complex (23). NAD(P)H-flavin reductase, *fre*, was also required in Dip 400 and it is likely that *fre* reducing the ferric iron of siderophores to ferrous iron (24). However, in severe iron restriction conditions, Dip250 and Dip400, these three genes became dispensable, specifically at Dip400 and the only source of iron was intracellular Fe-S clusters, *sufABCDES* (Fig 3.4). In iron-restricted conditions *E. coli* utilizes *suf* operon (25) and the operon is controlled by *iscR* (26). In agreement with this, we found that *Salmonella* utilizes *suf* system in iron restricted conditions and protein-protein interaction networks indicate that *suf* operon is under the control of *iscR* (Fig. S3.6). Further, γ-glutamyltranspeptidase, *ggt*, is an important enzyme in

glutathione metabolism and it is required in Dip250 and Dip400. It has been suggested that *ggt* plays a role in Fe-S cluster biosynthesis in eukaryote *Saccharomyces cerevisiae* (27). We speculate that *ggt* is participated in Fe-S cluster biosynthesis in *S. Typhimurium* in Dip250 and Dip400. Collectively, this demonstrates the role of Fe-S clusters and other genes in homeostasis of iron that directly or indirectly fuel Fenton reaction.

The fitness of the subunits of DNA polymerase V, *umuDC*, increased in iron-restricted conditions which is an indicator that *S*. Typhimurium lacking umuDC was grown better in iron-restricted conditions. In *E. coli*, a mutant strain lacking dnaE911, DdinB, and DumuDC is more resistant to killing by bactericidal antibiotics than wild-type; DNA polymerase III, IV, and V contribute to ampicillin-mediated cell death. Particularly, the generated ROS (hydroxyl radicals) following antibiotic treatment oxidizes guanine nucleotide pool to a mutagenic 8-oxo-deoxyguanosine (8-oxo-guanine) which results in lethal outcomes because incorporation of 8-oxo-guanine into DNA causes double-strand breaks (28). The essential genes *dnaEX* which encode subunits of DNA polymerase III and the conditionally essential genes *umuDC* which encode subunits of DNA polymerase V had increased fitness based on Tn-seq in iron-restricted conditions. The uncharacterized ORF *STM14_2428* is a neighbor of *STM14_2422* (*umuC*) had also increased fitness (Table 3.2), but it is unclear how *STM14_2428* deletion is in favor *S*. Typhimurium growth in iron-restricted conditions. Interestingly, two important conditionally essential genes, *guaB* and *purA* had increased fitness. These two genes catalyze the first step in the de novo synthesis of guanine and adenine from inosine 5'-phosphate (IMP). These genes may have a connection with 8-oxo-guanine, however we are devoid of evidence to support the role of *guaB* and *purA* in ROS pathways. Tn-seq shows that *S*. Typhimurium was grown better in iron-restricted conditions when *guaB* or *purA* deleted. These findings emphasize the role of DNA polymerases in ROS-mediated killing as deleting of these genes results of a better bacterial growth in iron restricted conditions.

We have several other genes that identified by Tn-seq with either increased or decreased fitness (Table 3.2 and Fig. 3.4). Some of theses genes may have connections with ROS formation and we briefly mention important ones. Entner-Doudoroff aldolase, *eda*, has a central role in sugar acid metabolism and detoxification of metabolites in *E. coli* (29). There are 6 mutants with increased fitness in bacterial membrane, outer membrane protein assembly (*nlpB*, *rfbB*, and *rfbH*), transmembrane transports (*sapA* and *smvA*), and a putative integral component of membrane (*STM14_0726*). RNA polymerase sigma-E factor, *rpoE*, and the serine endoprotease, *degS*, had reduced fitness and required for *S.* Typhimurium survival in Dip250 and Dip400. In *E. coli*, *rpoE* and *degS* are essential genes; *rpoE* is an extracytoplasmic factor that activates in response to envelope stress. The activation starts by unfolding outer membrane proteins (OMPs) and ends with proteolysis of anti-sigma-E factor by *degS* to free *rpoE* and initiate transcription (30, 31). This emphasizes the role of membrane in the process but uncovering connection between membrane and ROS generation requires future research.

3.4.8 ROS-independent essential genes may be better targets for antimicrobials

We believe that our finding will have a profound implication for the current antibiotic in clinical use and development of new antibiotics. We propose that ROS-independent essential genes may be better targets for antibiotics because of two main reasons. First, it has been shown that there are two opposing aspects of ROS-mediated killing mechanism. When ROS production is high, it would lead to facilitated killing of bacterial cells. On the contrary, when ROS production is low, it would lead to production of resistant mutants through mutagenic action of ROS on DNA (32). When *Salmonella* infects the host, the iron-restricted host niches would suppress the ROS-mediated killing mechanism and reducing overall killing effect by the antibiotics. However, depending on the iron restriction levels, it might allow production of low amount of ROS, facilitating bacterial survival through development of antibiotic resistant mutants. In contrast, there might be essential genes without ROS pathway contributing to

lethality (215 genes), and we speculate that these genes might serve as better targets for antibiotic development, because ROS production is not a part of their lethal processes and blocking the pathways will lead to killing, and the chance to develop resistant population via ROS action can be eliminated. Second, Tn-seq shows clearly that mutants of ROS-dependent essential genes can grow very slowly in iron-restricted conditions and the same phenomenon may happen in host because iron-restriction by host is a vital mechanism to combat the pathogen. As a result, it may be hard to completely eliminate and kill the bacteria by targeting ROS-dependent essential genes. Conversely, mutants of ROS-independent essential genes die immediately in iron-restricted or iron-replete conditions following the gene disruption. Thus, the possibility will be higher to eradicate a pathogen by targeting the ROS-independent genes.

A mechanism that bacteria exploits for antibiotic resistance is alteration of drug interaction site. Our results emphasize that the majority of genes of drug targets are ROS-dependent genes (Fig. 3.1). Prevalence of antibiotic resistant bacteria from clinical isolates due to mutations in drug targets have been rising. Mutations in a peptidoglycan synthesis gene *fts* which is target of β-lactams in *Haemophilus influenzae* cause resistance to antibiotics (33, 34). *E. coli* strains harboring mutations in *murA* are resistant to fosfomycin (35). UDP-N-acetylglucosamine enolpyruvyl transferase (MurA) is catalyze reaction in first step biosynthesis of peptidoglycan in bacterial cell wall and the protein is target of fosfomycin (36). Our Tn-seq shows that *murA* mutants did grow very well in iron-restricted conditions and the mutant had 14,981 reads in Dip400 but there were only 5 reads of this mutant in LB-III (Table 3.1). It has been reported that *Pseudomonas putida* develops intrinsic fosfomycin resistance due to present of a salvage pathway that bypasses *de novo* biosynthesis of MurA (37). Since *murA* is a ROS-depended essential gene, we reason that almost all *murA* mutants died in LB-III because of contribution of ROS in death process. However, in Dip400, reduced ROS formation and the salvage pathway biosynthesis of MurA caused *S*. Typhimurium to grow well.

Further, Fluoroquinolone-resistant bacteria are also present in clinical isolates due to mutations in drug targets, *gyrA*, *gyrB*, *parC*, *parE*, such as *Shigella flexneri* (38), *Salmonella* Typhi (39), and group B *Streptococcus* (40). Rifampin-resistant *Mycobacterium tuberculosis* isolates are associated with mutations in their targets, *rpoB* and *rpoC* (41, 42). Mutations in *rplC* contribute to *Staphylococcus aureus* resistance to linezolid in a clinical isolate (43). Because of mutations in *rplB*, *S. aureus* resistant-isolates detected *in vitro* (44). All together, these ROS-dependent essential genes, antibiotic targets, can mutate and alter the structure of corresponding proteins in order to evade lethal interactions with the antibiotics. Based on the algorithms that were used in this study for analyses, *gyrA* and *gyrB* were acted as ROS-depended and ROS-independent genes. However, there were a few ribosomal ROS-independent genes, usually do not interact with the drugs directly, contribute to antibiotic resistance via mutations in these genes including *rplD* (45), *rplV* (46), *rpsE* (47), and *rpsJ* (48).

An example of ROS-independent genes and target for antibiotic is colistin. Colistin (polymyxin E) is a last resort antibiotic for treatment of infections caused by multidrug resistant Gram-negative bacteria (49). This bactericidal drug interacts with the lipid A moiety of lipopolysaccharide (LPS) and ultimately causes membrane lysis (50). We show that colistin target genes are ROS-independent, *lpxABCDHK*. Over the last 60 years, colistin has been using for fighting the infectious diseases with some hesitation of its use due to toxicity. Thus, it has been believed that colistin is still active and bacterial resistance is low because of its infrequent use. Our Tn-seq indicates that disruption of LPS is lethal in *S.* Typhimurium and there is no contribution of ROS in death process via LPS protein damage. However, a study demonstrated that colistin induce *Acinetobacter baumannii* killing through ROS production (51). This takes us back to the first point of the model, common antibiotic killing mechanism via ROS. Although this model is widely accepted, a few studies challenged it (52, 53). Thus, contrary to our findings will be expected due to differences in applied methodology. Our used method in

this work is unique and incomparable to the methods of the studies engaged in ROS experiments. The precession and specificity of our Tn-seq is very high. The genomic DNA extracts from the exposed conditions amplified by a linear PCR and followed by an exponential PCR. This PCR product amplified again on the flow cell of Illumina sequencing to form clusters and the DNA sequence of a strand will be utilized if passed the DNA sequencing quality control. We tried to reproduce and confirm our findings either with PCR or CFU measurements, but there was no success. Tn-seq indicates that mutants of ROS-dependent essential genes were not die following disruption; they can not form visible colonies on agar plates and their optical density indiscernible. We generated these mutants on a filter paper put on an agar plate followed by 24 h growth on agar plates contain appropriate antibiotics. After several month of storage in 7% DMSO at -80°C, the mutants were not dead and getting read counts with Tn-seq in iron-restricted conditions are indicator that these mutants grow very slowly. However, mutants of ROS-dependent genes die when iron is replete due to ROS.

3.5 Conclusion

In this work we exploited Tn-seq to elucidate the genes that are ROS dependent. Our powerful Tn-seq approach indicated that when transposon mutant cultures treated with an iron chelator, the mutants of one-third of essential genome of *Salmonella* Typhimurium did not die and could grow slowly, however these mutants died in absences of the iron chelator. Based on this observation, we concluded that the iron chelator minimized ROS formation via downregulation of Fenton reaction, as a result, one-third of essential genes did grow likely because ROS is present in their death process. Eventually, we call this one-third of essential genome ROS-dependent essential genes, and the rest of essential genome is ROS-independent essential genes. The result is fitting to known model of common ROS-mediated antibiotic killing in bacteria and we further expand this model beyond antibiotic target genes. Strikingly, the targets of almost all antibiotics in clinical use are ROS-dependent essential genes. We

propose that ROS-independent essential genes are better targets to develop new antimicrobials, as the cell die immediately following gene disruption. In addition to these, we identified the dynamics of conditionally essential genes that mediate *S.* Typhimurium survival in a gradient iron-restricted conditions. The finding is exclusively based on a high resolution Tn-seq. We are planning to study phenotype of essential genes through a combination of Tn-seq and CRISPRi in future research.

3.6 Materials and Methods

3.6.1 Measurement of *S.* Typhimurium growth under 2,2`-Dipyridyl

A single colony of *S.* Typhimurium was inoculated into 2 ml LB broth medium in a 5 ml tube and incubated overnight (~16 h). Freshly prepared LB broth media supplemented with different concentrations of Dip were inoculated with *S.* Typhimurium overnight culture at a 1:200 dilutions. The cultures were immediately added into a 96-well microplate (200 µl/well) and incubated in a Tecan Infinite 200 microplate reader at 37°C, with shaking amplitude of 1.5 mm, and shaking duration of 5 s, and OD_{600} was measured every 10 min. After 24 h incubation, the data were collected from which lag time phase, growth rate, and maximum OD_{600} were calculated for each concentration using GrowthRates script (54).

3.6.2 Construction of Tn5 mutant libraries

Transposon mutant libraries were prepared as previously described by Karash et al., (20). Briefly, *Salmonella* enterica serovar Typhimurium ATCC 14028S were mutagenized by biparental mating using *Escherichia coli* SM10 λ*pir* carrying a pBAM1 transposon-delivery plasmid vector (55) as the donor. An equal volume of overnight growth cultures of the donor and recipient bacteria (*S.* Typhimurium 14028s) were washed with 10 mM $MgSO_4$ and concentrated on the nitrocellulose filter, which was then incubated for 5 h at 37°C on a surface of LB agar plate. After the conjugation, the cells were washed with 10 mM $MgSO_4$ and plated

on LB agar plates contain appropriate antibiotics. The plates were grown at 37°C for 24 h. Then, colonies were scrapped off, added into LB broth supplemented with 7% DMSO, and stored at −80°C in aliquots. We constructed two mutant libraries, A and B. Each library contain approximately 325,000 mutants.

3.6.3 Mutant library selection for Tn-seq

An aliquot of transposon library was thawed at room temperature and diluted 1:10 in LB broth. The library was incubated at 37°C with shaking at 225 rpm for an hour and then washed twice with PBS. The library-A was inoculated to 20 ml LB broth in a 300 ml flask and LB supplemented with either 100 or 150 μM Dip (LB-I, Dip100, and Dip150, respectively), seeding CFU was 3.5 x 10^6 per ml. We also had a condition without growth (LB-II), the library-A was directly subjected to Tn-seq after activation and washing. To make a super saturated mutant library, library-A was combined with library-B and called library-AB. Library-AB treated as mentioned above and was inoculated to 20 ml LB broth in a 300 ml flask and LB supplemented with either 250 or 400 μM Dip (LB-III, Dip250-I, Dip250-II, and Dip400, respectively), seeding CFU was 8 x 10^6 per ml. The Dip100, Dip150, Dip250-I, Dip250-II, and Dip400, were incubated at 37°C with shaking at 225 rpm in a dark and humidity controlled incubator until the cultures reach mid-log phase, OD_{600} of ~2.7. Then, the cultures were immediately centrifuged and stored -20°C for downstream analysis.

3.6.4 Preparation of Tn-seq libraries for HiSeq sequencing

Tn-seq libraries preparation were performed as previously described by Karash et al., (20). Briefly, genomic DNA was extracted for each of selected conditions using DNeasy Blood & Tissue kit (Qiagen), and quantified using Qubit dsDNA RB Assay kit (Invitrogen). To remove the cointegrates, genomic DNA was digested with PvuII-HF (New England Biolabs), and purified with DNA Clean & Concentrator-5 kit (Zymo Research). Then, a linear PCR

extension was performed using Tn5-DPO (5'-AAGCTTGCATGCCTGCAGGTIIIIICTAGAG GATC-3'). The PCR reaction was performed in a 50 µl contained Go Taq Colorless Master Mix (Promega), 20 µM Tn5-DPO primer, 100 ng gDNA, MQ-H_2O. The PCR cycles were consisted of 95°C for 2 min, followed by 50 cycles at 95°C for 30 sec, 62°C for 45 sec, and 72°C for 10 sec. The PCR product was purified with DNA Clean & Concentrator-5 kit. The C-tailing reaction was conducted with terminal transferase (TdT) buffer (New England Biolabs), $CoCl_2$, dCTP, ddCTP, TdT and the purified linear PCR product. The mixture was incubated at 37°C for 1 h and followed by 10 min at 70°C. The C-tailed product was purified. Next, the exponential PCR was performed with P5-BRX-TN5-MEO primer, AATGATACGGCGACCA CCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNAG-BARCOD-CCTAGGCGGCCTTAATTAAAGATGTGTATAAGAG and P7-16G primer, CAAGCAGA AGACGGCATACGAGCTCTTCCGATCTGGGGGGGGGGGGGGGG. The PCR reaction was performed in a 50 µl contained Go Taq Green Master Mix, P5-BRX-TN5-MEO primer, P7-16G primer, purified C-tailed genomic junctions, and MQ-H_2O; the PCR cycles were consisted of 95°C for 2 min, followed by 30 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 20 sec, with the final extension at 72°C for 5 min. Then, the 50 µl PCR products were run on an agarose gel and the DNA fragment of size 325 – 625 bp was cut the DNA was extracted using Zymoclean Gel DNA Recovery kit (Zymo Research). The DNA libraries were quantified using Qubit dsDNA RB Assay kit. The libraries were combined and sequenced on a flow cell of HiSeq 3000 using single end read and 151 cycles (Illumina) at the Center for Genome Research & Biocomputing in Oregon State University.

3.6.5 Analysis of Tn-seq data

The Hi-Seq sequence results were downloaded onto High Performance Computing Center (AHPCC) at the University of Arkansas. The libraries were de-multiplexed using a custom Python script. The script searched for the six-nucleotide barcode for each library and

mismatch did not allowed. The transposon genomic junctions were extracted by using Tn-Seq Pre-Processor (TPP) tool (56). The TPP searched for the 19 nucleotide inverted repeat (IR) in a fixed sequence window and identified five nucleotides (GACAG) at the end of the IR sequence, one nucleotide mismatch was allowed. The genomic junctions that start immediately after GACAG were extracted and the C-tails were removed. The junction sequences of less than 20 nucleotides were removed and remaining junction sequences were mapped to the *Salmonella* enterica serovar Typhimurium 14028S genome and plasmid using BWA-0.7.12 (57). The TPP was counted number of total sequences reads after filtering, number of mapped read, and number of unique insertions in the library.

3.7.6 Identification of essential genes

LB-I, LB-II, and LB-III were analyzed to identify the essential genes in *S.* Typhimurium. We used two different tools for Tn-seq essential gene analysis. First, TRANSIT (56) analysis of essentiality on gaps in entire genome was used, tn5gaps algorithm. The 5% of N-terminal and 10% of C-terminal of open reading frames (ORF) were removed and even insertions with only one reads were considered for the analysis. The gene was considered essential if its p value ≤ 0.05. Second, Tn-Seq Explorer (58), was used for essential gene analysis by applying a 550 window size. The 5% of N-terminal and 10% of C-terminal ORFs were removed and even insertions with only one reads were considered for the analysis. The gene was considered essential if its Essentiality Index was ≤ 2. Then, the essentiality analysis results by both methods were combined. Finally, to consider a gene essential for growth on LB agar or LB broth should has these three criteria: (i) the gene is essential in LB-III by Tn-Seq Explorer analysis (ii) the gene is essential in LB-III by TRANSIT analysis (iii) the gene is essential in at least 5 of the 6 analysis that was performed for the LB-I, LB-II, and LB-III by the two analysis tools (Fig. S4). We made an exception of 17 genes to be considered essential. Instead of 5 essential requirements, we changed to 4. This exception was based on the other

analysis for the same libraries but under different growth conditions.

3.6.7 Identification of conditionally essential genes (gene fitness measurement)

The conditionally essential genes for all iron-poor conditions were analyzed by using TRANSIT, resampling option. The LB-I was input for Dip100 and Dip150; the LB-II was input for Dip250-I, Dip250-II, and Dip400. The normalization method was Trimmed Total Reads (TTR) and 10,000 samples were used for the analysis. The 5% of N-terminal and 10% of C-terminal of ORFs were removed and the gene was considered conditionally essential if the its *p* value was ≤ 0.05. Each iron-poor condition has its own set of genes that were required for to resist the condition. To make a comprehensive list the *S*. Typhimurium that are required for iron poor condition, specifically for Dip400 and including Dip250-I, Dip250-II, Dip-150, and Dip100, the gene was considered required if its p value was ≤ 0.05 in Dip400 or other Dip conditions and its log2 fold change (log2FC) was negative.

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3.8 Author Contributions

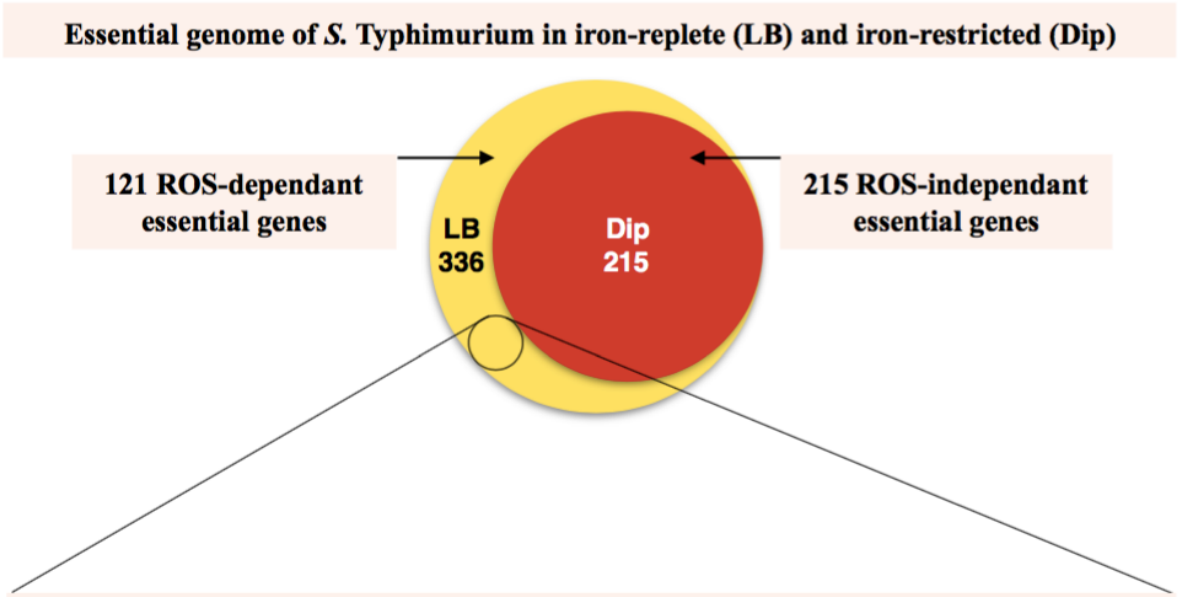
Conceived and designed the experiments: YK SK. Performed the experiments, analyzed the data, wrote the manuscript: SK. Revised the manuscript: YK SK.

3.9 Figures and Tables

Essential genes have increased insertions and read counts in iron-restricted conditions
(LB: iron-replete, Dip: iron-restricted, I: insertion, R: read count, fitness: Log2 fold change)

		LB-III		Dip250		Dip400			
Biological Process	**Gene**	**I**	**R**	**I**	**R**	**I**	**R**	Fitness	p value
Cell division	***mukB***	3	9	6	184	5	120	3.73	0.025
Cell membrane	***yfiO***	1	3	4	129	6	180	5.9	0.0015
Cell wall biosynthesis	***ftsI***	1	1	1	0	5	210	7.71	0.0137
Cell wall biosynthesis	***mrdB***	0	0	5	24	5	98	4.36	0.0087
Cell wall biosynthesis	***murA***	1	5	3	45	4	14981	11.55	0.0008
Coenzyme A biosynthetic process	***dfp***	0	0	1	0	6	152	4.33	0.0001
Coenzyme A biosynthetic process	***coaA***	0	0	5	185	5	125	4.45	0.0021
DNA replication	***dnaE***	0	0	3	9	8	155	4.35	0.0004
DNA replication	***dnaX***	0	0	2	2	5	65	3.81	0.0085
DNA replication	***nrdA***	0	0	4	141	6	286	5.39	0.0006
DNA replication	***parC***	1	1	1	15	4	98	6.61	0.0458
DNA replication	***parE***	0	0	4	22	4	125	5.01	0.0287
Fatty acid metabolic process	***fabH***	0	0	3	42	5	74	3.97	0.006
Glutamine metabolic process	***glmS***	0	0	4	126	4	98	4.36	0.0058
Glutamine metabolic process	***pyrG***	1	7	4	5744	4	185	4.72	0.0462
Phospholipid biosynthetic process	***pssA***	1	7	4	88	5	139	4.31	0.016
Protein transport	***secY***	1	1	5	49	5	19	4.25	0.0139
Protein transport	***yidC***	1	1	6	165	4	144	7.17	0.0241
Transcription	***gyrA***	0	0	2	2	3	106	4.78	0.0253
Transcription	***gyrB***	0	0	2	0	3	152	5.29	0.0308
Transcription	***rpoB***	0	0	12	164	13	392	4.96	0.0000
Transcription	***rpoC***	1	1	9	186	10	150	7.23	0.0000
Translation	***glyS***	0	0	2	31	7	250	5.2	0.0007
Translation	***ileS***	0	0	1	2	4	84	4.47	0.0284
Translation	***infB***	0	0	2	12	5	57	3.64	0.0057
Translation	***proS***	0	0	2	0	5	71	3.92	0.0088
Translation	***rplB***	0	0	3	134	6	74	3.97	0.0074
Translation	***rplC***	1	1	1	6	4	38	5.25	0.046
Translation	***rpsD***	0	0	1	13	3	242	5.94	0.0284
Translation	***thrS***	2	4	6	5855	10	207	5.69	0.0001
Translation	***valS***	2	3	4	2446	7	147	5.61	0.0031
Translation	***glyQ***	0	0	2	55	3	133	4.79	0.0071
Unknown	***yfgM***	1	2	3	7	5	98	5.61	0.0077

Table 3.1. *Salmonella* Typhimurium transposon mutants were grown in iron-replete (LB) and iron-restricted conditions (Dip). Tn-seq identified these genes as essential in LB but not in Dip conditions. Dip is abbreviation of iron chelator 2,2`-Dipyridyl in µM. As the concentration of Dip increased, the insertion and read counts increased. The fitness of these 33 genes increased in Dip400, 400 µM of iron chelator. Gene fitness is Log2 fold change in sequence reads, Dip400 vs LB.



Gene	Read counts		Gene Fitness (Log2FC)	Antibiotic	Target
	LB-III	Dip400			
ftsI	1	210	7.71	β-lactams	Cell wall synthesis
gyrA	0	106	4.78	Fluoroquinolones	DNA synthesis
gyrB	0	152	5.29	Fluoroquinolones	DNA synthesis
murA	5	14981	11.55	Fosfomycin	Cell wall synthesis
parC	1	98	6.61	Fluoroquinolones	DNA synthesis
parE	0	125	5.01	Fluoroquinolones	DNA synthesis
rplB	0	74	3.97	Aminoglycoside	Protein synthesis
rplC	1	38	5.25	Aminoglycoside	Protein synthesis
rpoB	0	392	4.96	Rifampin	RNA synthesis
rpoC	1	150	7.23	Rifampin	RNA synthesis
rpsD	0	242	5.94	Aminoglycoside	Protein synthesis

Fig 3.1. ROS-dependent essential genes, ROS-independent essential genes, and antibiotics targets. *Salmonella* Typhimurium transposon mutants were grown in LB media (iron-replete) and LB supplemented with an iron chelator Dip (2,2`-Dipyridyl). Mutants of ROS-dependent genes have significant read counts in iron-restricted. Gene fitness was calculated based on Tn-seq read counts of iron-replete and iron-restricted condition. The genes of some mutants with increased fitness are antibiotic targets.

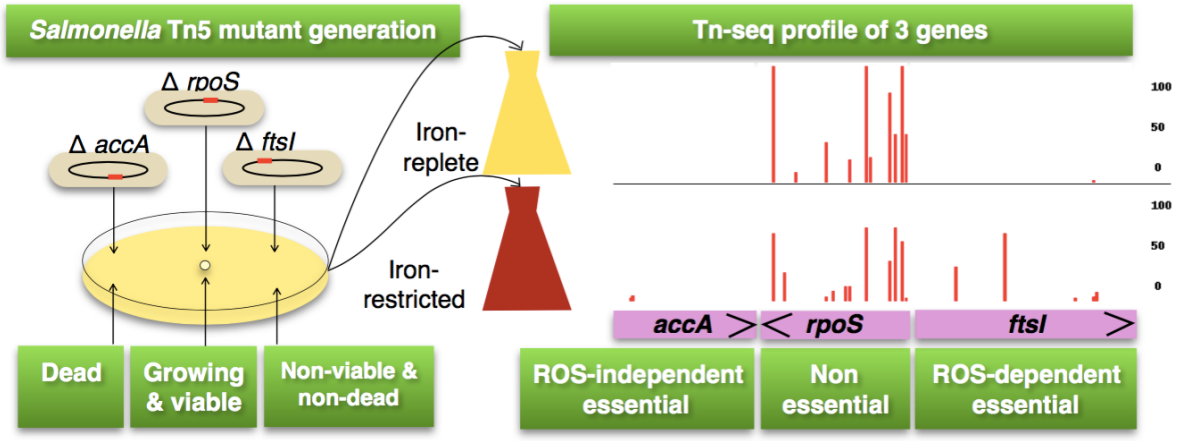


Fig 3.2. Identification of ROS-dependent essential genes by Tn-seq. *Salmonella* Typhimurium Tn5 mutants generated and the library inoculated to iron-replete media (LB broth) and iron-restricted media (LB supplemented with an iron chelator 2,2`-Dipyridyl). The cultures were grown till mid-log phase and then Tn-seq was identified essential and conditionally essential genes as well as fitness of all genes. The essential genes with increased fitness in iron-restricted conditions are ROS-dependent. The genes that do not tolerate insertions in both conditions are ROS-independent essential genes. The numbers on right (0-200) are read counts and each red line represent a unique insertion.

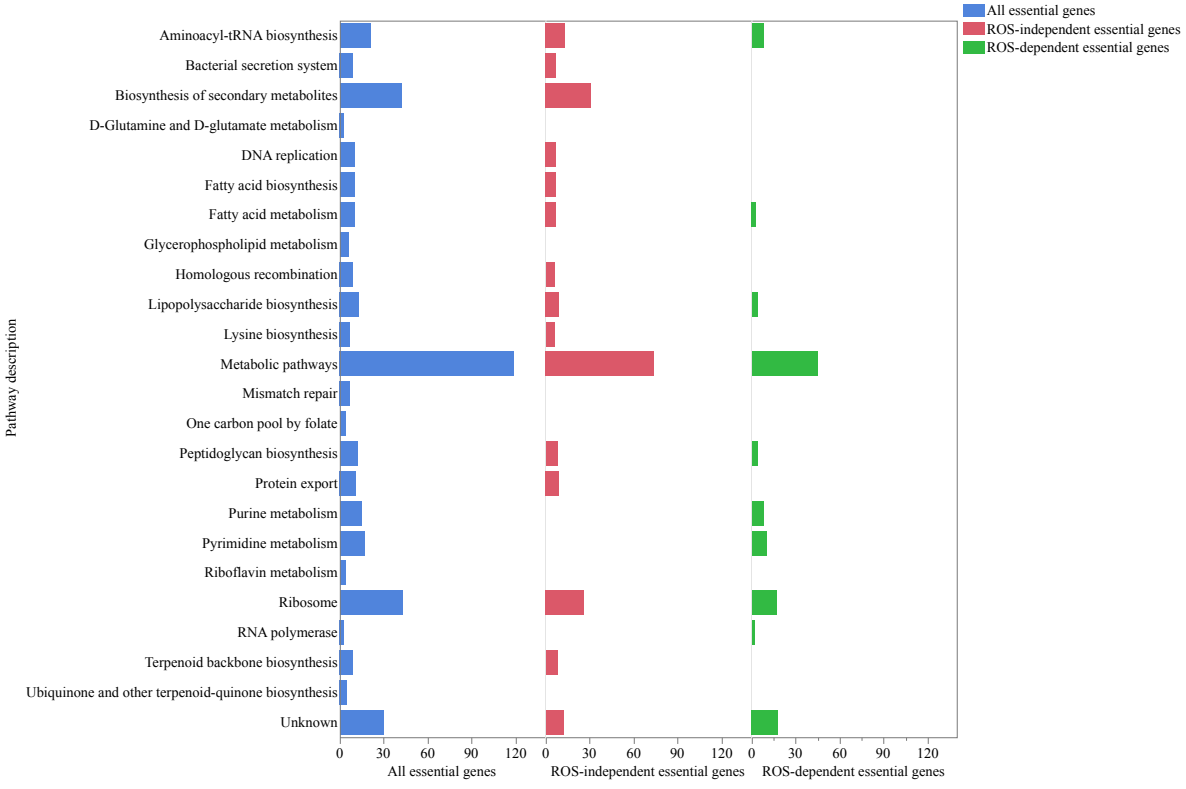


Fig 3.3. ROS-dependent essential pathways in *Salmonella* Typhimurium identified by Tn-seq. Transposon libraries were grown in iron-replete and iron restricted conditions. Essential genes identified for both conditions. The genes that were essential in iron-replete condition but not in iron-restricted conditions were considered ROS-dependent. KEGG pathway analysis was used for pathways description.

Conditionally essential genes with increased fitness in iron-restricted condition

Biological Process	Gene	Read Count LB-III	Read Count Dip400	Gene Fitness Log2FC	p value
Amino-acid biosynthesis	***trpE***	1,930	3,991	1.05	0.047
DNA repair, SOS response	***umuD***	131	542	2.05	0.0343
DNA repair, SOS response	***umuC***	1,492,158	6,078,627	2.03	> 0.05
hypothetical protein	***STM14_2428***	1,061,042	5,444,922	2.36	> 0.05
Gluconeogenesis	***STM14_2709***	2,286	4,939	1.11	0.0256
Nucleotide biosynthesis	***guaB***	559	1,359	1.28	0.021
Nucleotide biosynthesis	***purA***	410	1,585	1.95	0.0444
Integral component of membrane	***STM14_0726***	22	330	3.9	0.0329
Outer membrane protein assembly	***nlpB***	296	997	1.75	0.0221
Outer membrane protein assembly	***rfbB***	6,652	10,723	0.69	0.0412
Outer membrane protein assembly	***rfbH***	3,962	8,777	1.15	0.0184
Transmembrane transport	***sapA***	349	874	1.32	0.0175
Transmembrane transport	***smvA***	1,862	3,624	0.96	0.0239
TCA cycle	***acnB***	1,925	4,873	1.34	0.0193
TCA cycle	***icdA***	19	474	4.64	0.0000
Carbohydrate metabolism	***eda***	25	308	3.62	0.0279
Translation	***tuf_1***	58	450	2.95	0.0255
Putative regulator	***STM14_3217***	22	229	3.38	0.018

Table 3.2. *Salmonella* Typhimurium transposon mutants were grown in iron-replete (LB) and iron-restricted conditions (Dip). Tn-seq identified conditionally essential genes with increased fitness in Dip conditions. Dip is abbreviation of iron chelator 2,2`-Dipyridyl in μM. As the concentration of Dip increased, the insertion and read counts increased. The fitness of these 16 genes increased in Dip400, 400 μM of iron chelator. Gene fitness is Log2 fold change in sequence reads, Dip400 vs LB.

Tn-seq identified conditionally required essential genes for iron-restricted conditions in *S.* Typhimurium, gene with reduced fitness, p < 0.05

Biological process	Dip100	Dip150	Dip250	Dip400
Cellular response to misfolded protein			*degS*	*degS*
Iron ion homeostasis	*fepD*	*fepD*	*fepD*	
Iron ion homeostasis			*fre*	
Glutathione biosynthetic process			*ggt*	*ggt*
Response to osmotic stress			*osmE*	*osmE*
Phosphate ion transport			*pstB*	*pstB*
Transcription initiation			*rpoE*	*rpoE*
Unknown			*STM14_4330*	*STM14_4330*
Iron-sulfur cluster assembly			*sufA*	*sufA*
Iron-sulfur cluster assembly			*sufB*	*sufB*
Iron-sulfur cluster assembly			*sufC*	*sufC*
Iron-sulfur cluster assembly			*sufD*	*sufD*
Iron-sulfur cluster assembly			*sufS*	*sufS*
Siderophore transport		*tonB*	*tonB*	
Regulation of transcription			*yfhP (iscR)*	*yfhP (iscR)*
Unknown			*ygjQ*	*ygjQ*
Iron-sulfur cluster assembly			*ynhA (sufE)*	*ynhA (sufE)*
Detoxification of zinc ion		*zntA*	*zntA*	*zntA*

Fig 3.4. Dynamics of conditionally essential genes that mediate Salmonella Typhimurium survival in iron-restricted conditions. Transposon libraries were inoculated to LB broth media supplemented with 100 µM iron chelator 2,2`-Dipyridyl (Dip100), 150 µM Dip (Dip150), 250 µM Dip (Dip250), or 400 µM Dip (Dip400). The cultures were grown till mid-log phase. Tn-seq calculated gene fitness by comparing Dip conditions with a Dip-free condition.

3.10 Supplementary Information

	Seeding CFUs/ml	CFUs/ml at mid-log phase	Time to reach mid-log phase h:min	OD600
Library-A	**325,000 mutant colonies recovered on 50 LB agar plates**			
LB-I	3,500,000	117,000,000	5	2.630
Dip100	3,500,000	190,000,000	5:35	2.610
Dip150	3,500,000	164,000,000	6:05	2.565
Library-AB	**325,000 mutant colonies recovered on 50 LB agar plates + Library-A (total: 650,000 mutants)**			
LB-III	8,000,000	700,000,000	5:30	2.567
Dip250-I	8,000,000	2000,000,000	10	2.446
Dip250-II	8,000,000	2010,000,000	10	2.462
Dip400	8,000,000	550,000,000	24	1.843

Table S3.1. Transposon inoculum densities and CFUs at mid-log phase. The seeding CFUs of all cultures counted following inoculation at time zero and at the mid-log phase when the growth stopped. OD600 measurements were used to monitor the growth. LB is broth free of Dip. Dip is abbreviation of iron chelator 2,2`-Dipyridyl in µM. The number with Dip is concentration of Dip.

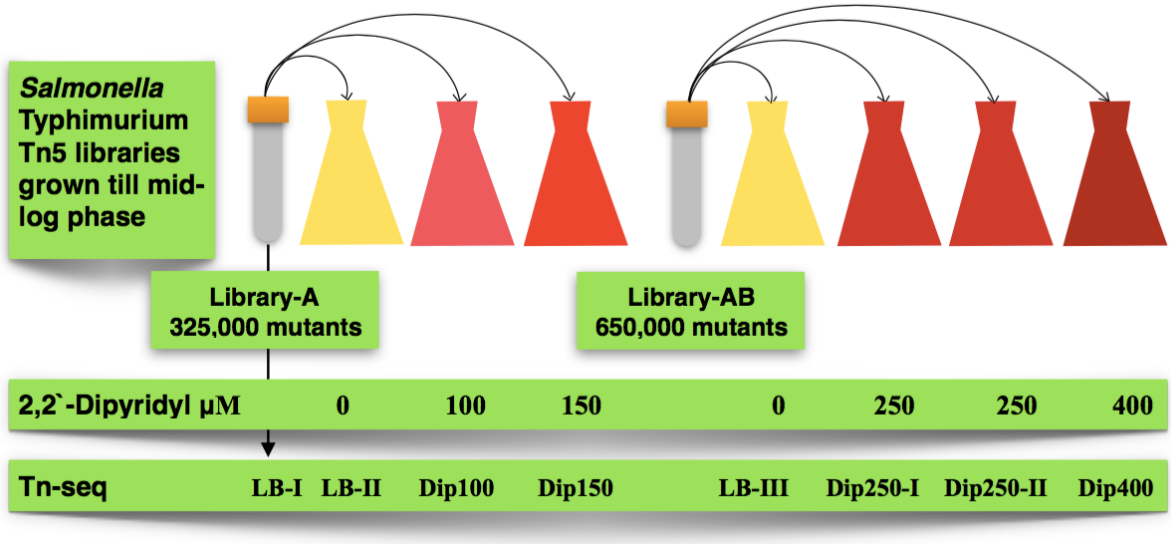


Fig S3.1. Schematic representation of study design. Transposon library-A inoculated to LB broth (LB-II) or the LB contained either 100 µM iron chelator Dip (Dip100) or 150 µM Dip (Dip150). LB-I was library-A that subjected to Tn-seq without growth. Transposon library-B inoculated to LB broth (LB-III) or the LB contained either 250 µM Dip (Dip250) or 400 µM Dip (Dip400). The cultures were grown till mid-log phase and then subjected to Tn-seq.

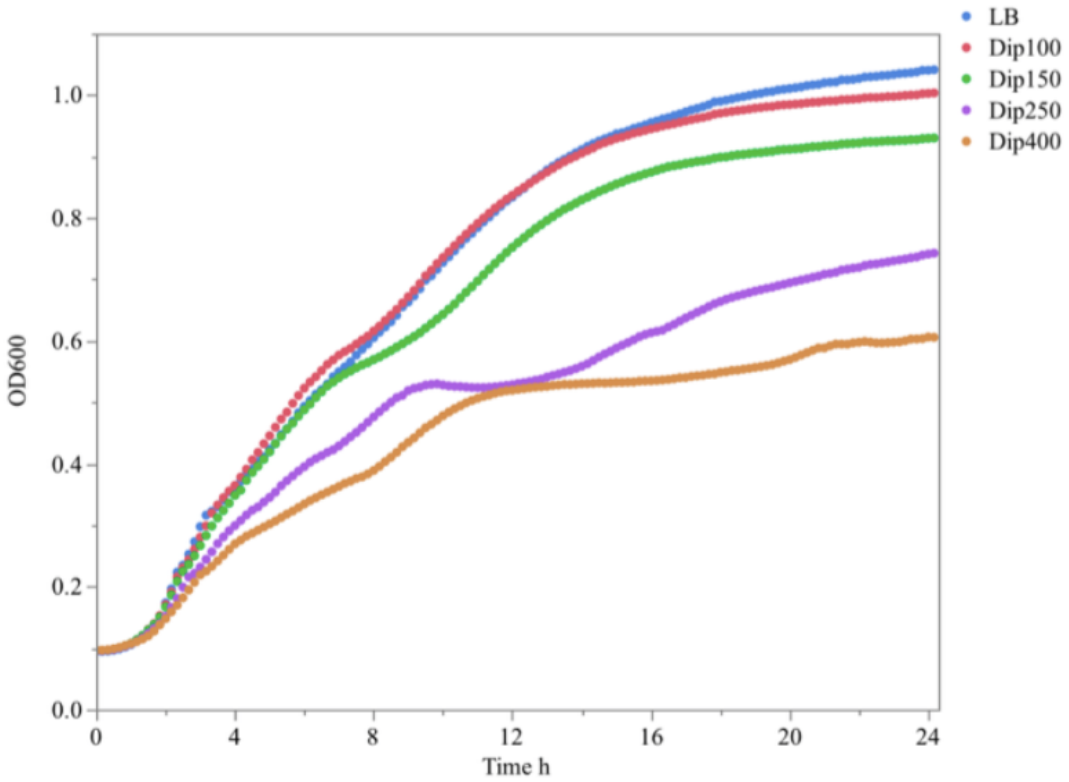


Fig S3.2. Effect of 2,2`-Dipyridyl (Dip) on S. Typhimurium growth. An overnight culture diluted 1:200 in LB broth supplemented with 100 µM Dip (Dip150), 150 µM Dip (Dip150), 250 µM Dip (Dip250), 400 µM Dip (Dip400), or Dip free (LB). The cultures were added to a 96-well plate and directly incubated at 37oC in a plate reader, reading OD600 every 10 minuets.

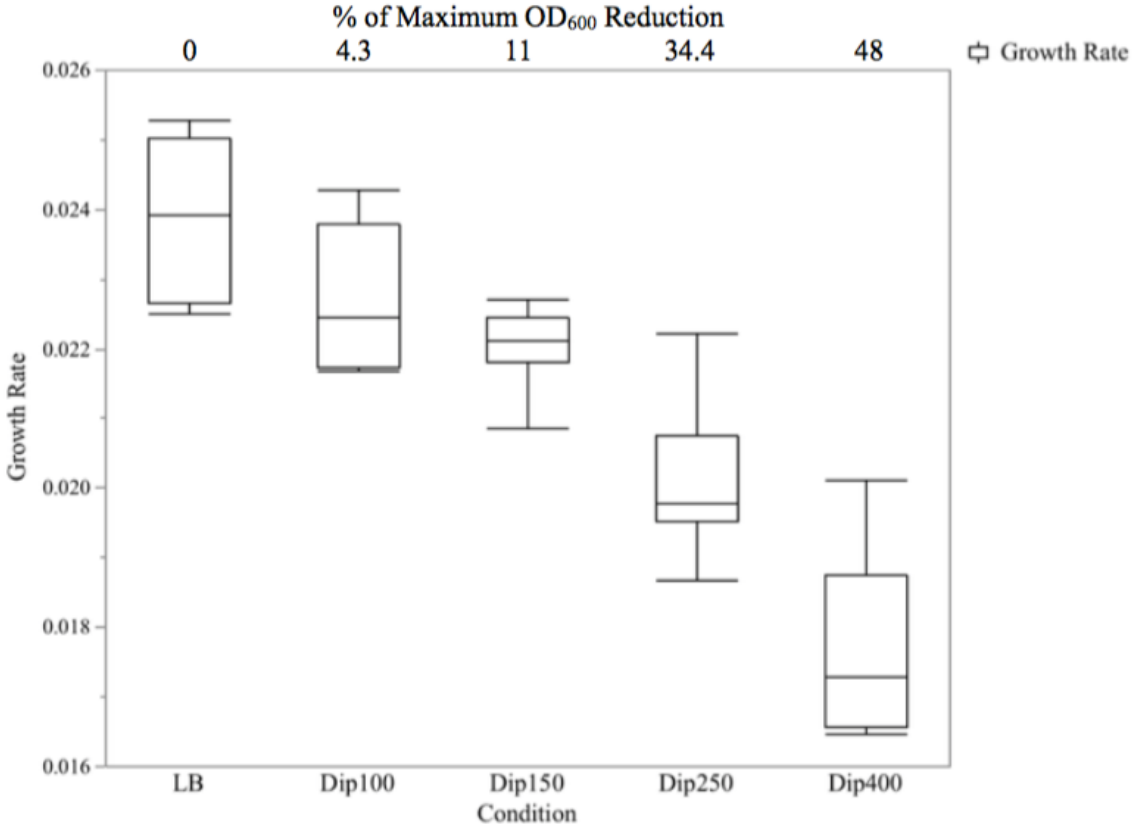
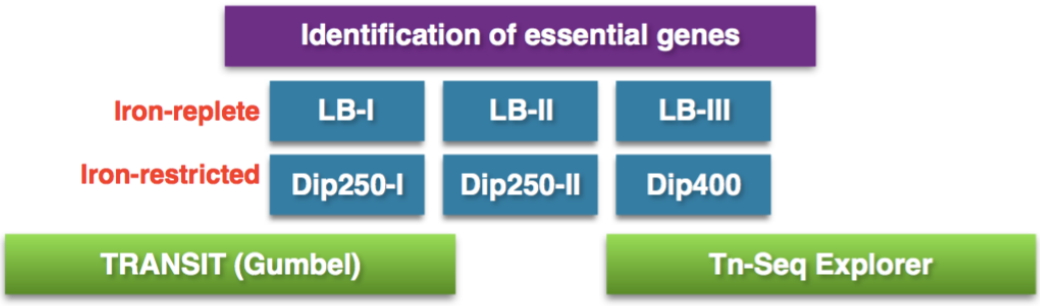


Fig S3.3. Effect of 2,2`-Dipyridyl (Dip) on S. Typhimurium growth rate and cell density. An overnight culture diluted 1:200 in LB broth supplemented with 100 µM Dip (Dip150), 150 µM Dip (Dip150), 250 µM Dip (Dip250), 400 µM Dip (Dip400), or Dip free (LB). The cultures were added to a 96-well plate and directly incubated at 37oC in a plate reader, reading OD600 every 10 minuets. The maximum OD600 reduction is shown as a percentage.

Number of mutants, reads, and insertions for Tn-seq selections in chromosome of S. Typhimurium

	Total reads with Tn5	Extracted reads >20bp	Mapped Reads	Unique Insertions	Mean genomic length bp
Library-A	**325,000 mutant colonies recovered on 50 LB agar plates**				
LB-I	18,225,644	14,437,819	12,289,451	115,784	93.8
LB-II	38,808,640	31,728,005	25,223,444	125,449	92.9
Dip100	25,788,698	21,034,947	16,991,894	117,474	93.0
Dip150	36,677,408	29,905,496	24,364,738	121,132	93.1
Library-AB	**325,000 mutant colonies recovered on 50 LB agar plates + Library-A (total: 650,000)**				
LB-III	57,779,778	47,575,248	39,248,662	193,728	90.6
Dip250-I	29,832,849	25,082,465	21,096,630	179,562	90.1
Dip250-II	35,439,669	28,119,351	23,104,233	181,534	89.1
Dip400	30,028,187	26,382,625	23,135,546	169,666	91.0
Total	272,580,873	224,265,956	185,454,598	1,204,329	91.7

Table S3.2. Sequence read counts used in this study. Total reads with Tn5 represent the sequence reads that passed the quality control and had sequence of Tn5. Extracted reads > 20 bp represent sequence reads that had trimmed C-tail (if present) and their length were above 20 nucleotides. Number of mapped reads, unique insertions in chromosome with mean length of mapped reads are shown. LB is broth free of Dip. Dip is abbreviation of iron chelator 2,2`-Dipyridyl in μM. The number with Dip is concentration of Dip.



			A	LB-III Essentiality Index < 3
B	LB-III Essential p < 0.05			
		C		
	The gene has to be essential in at least 5 of the six essentiality analyses			

Example-1

Gene	LB-I	LB-II	LB-III		LB-I	LB-II	LB-III	Call
murA	E	E	E		0	0	0	E
dapD	NE	E	E		1	1	0	E
acpP	NE	NE	E		2	1	1	NE
gmhA	NE	NE	E		5	1	0	NE
ssb	E	NE	E		3	0	1	NE
cydC	E	E	E		3	0	1	E

Gene	Dip250-I	Dip250-II	Dip400		Dip250-I	Dip250-II	Dip400	Call
murA	NE	E	E		4	2	4	NE
dapD	E	NE	E		0	2	2	E
acpP	NE	E	NE		2	1	2	NE
gmhA	NE	E	NE		1	0	1	NE
ssb	NE	NE	E		4	2	3	NE
cydC	E	E	E		2	4	5	NE

Fig S3.4. Algorithms utilized for essential gene identification. Two tools were used for essential gene analysis, TRANSIT (Gumbel) and Tn-Seq Explorer. LB-I, LB-II, and Lb-III were analyzed separately by both tools for identification of essential genes. The gene was considered essential if 5 out of 6 analyses were essential (E) or essentiality index (EI) < 3. LB-I was transposon library inoculum subjected to Tn-seq without growth. LB-II and LB-III were grown in LB broth till mid-log phase. Dip is abbreviation of iron chelator 2,2`-Dipyridyl in µM. The number with Dip is concentration of Dip.

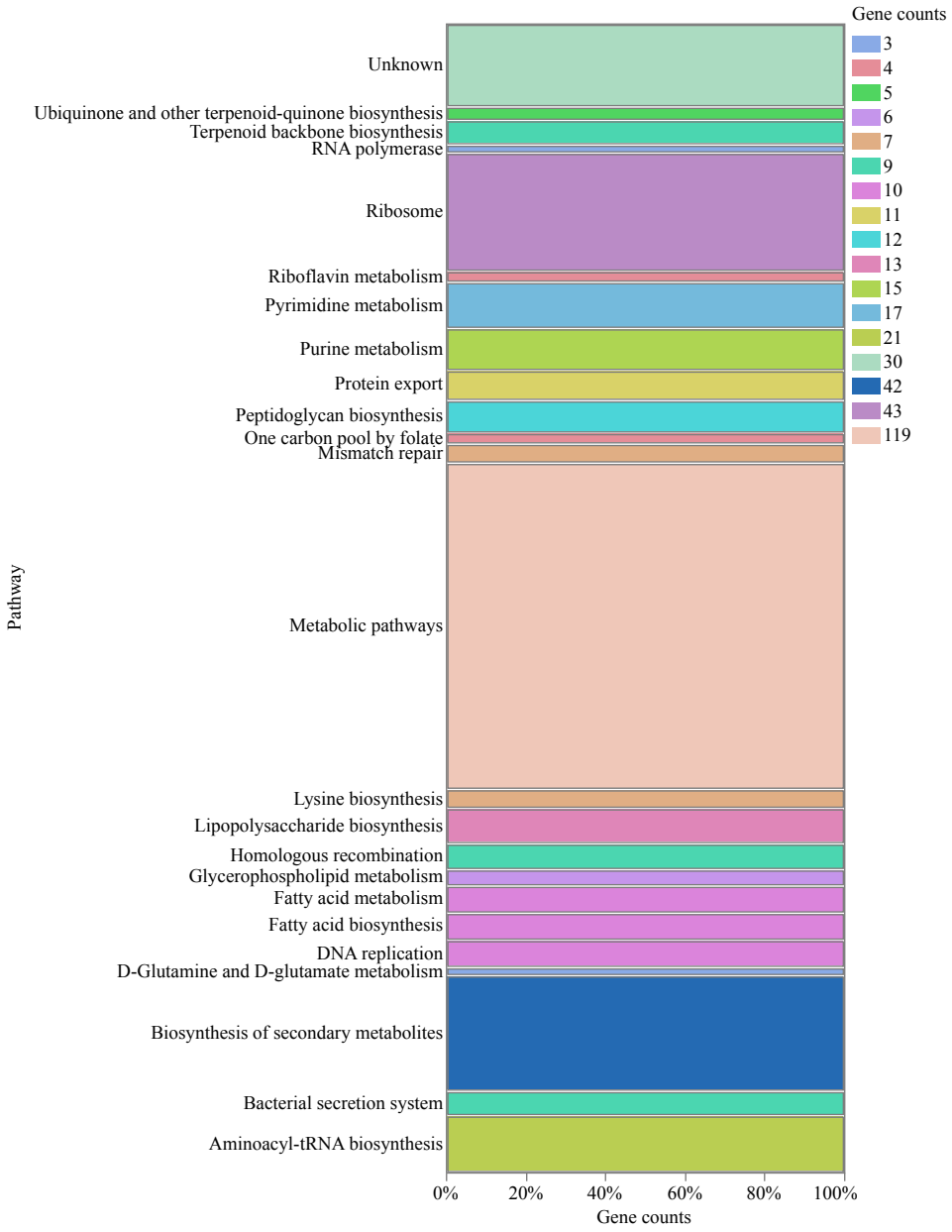


Fig S3.5. Required essential pathways of *Salmonella* Typhimurium in rich media. Tn-seq libraries were grown in LB broth media till mid-log phase and on LB agar. The total number of essential genes were 336 and KEGG pathway analysis categorized them to 23 essential pathways.

	LB-III	Dip250-I	Dip250-II	Dip400
Total reads	29,404,560	16,905,129	16,936,127	15,652,549
Total insertions	118,086	118,035	114,400	113,328
umuC (STM14_2422)	1,491,858	1,715,347	24,472	2,232,906
STM14_2428	1,061,042	1,483,718	23,694	2,000,137
Sum of reads in 2 mutants	2,552,900	3,199,065	48,166	4,233,043
% of reads consumed by 2 mutants	8.69	18.93	0.29	27.05
# of reads/insertion	227	116	147	100

Table S3.8. Bias in read sequencing is in favor of LB (iron-replete) conditions. Total sequencing reads and insertions in ORFs are shown. Sequencing reads consumed by two mutants, *umuC* and *STM14_2428* are also shown. An insertion in iron-replete (LB) has a chance to get 227 sequence reads but this reduced to 100 reads in iron-restricted condition (Dip400).

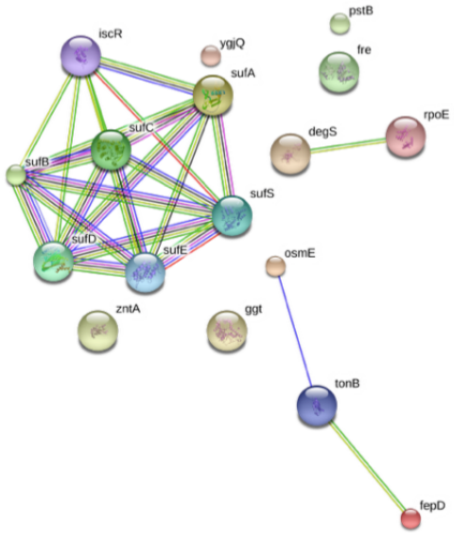


Fig S3.6. Protein-protein interactions of conditionally essential genes that mediate *Salmonella* Typhimurium survival in iron-restricted conditions. Transposon mutant libraries inoculated to LB (free of iron chelator Dip) and LB supplemented with various concentrations of Dip. The cultures were grown till mid-log phase. The gene fitness measured by Tn-seq. The interaction analysis conducted by using default settings of STRING.

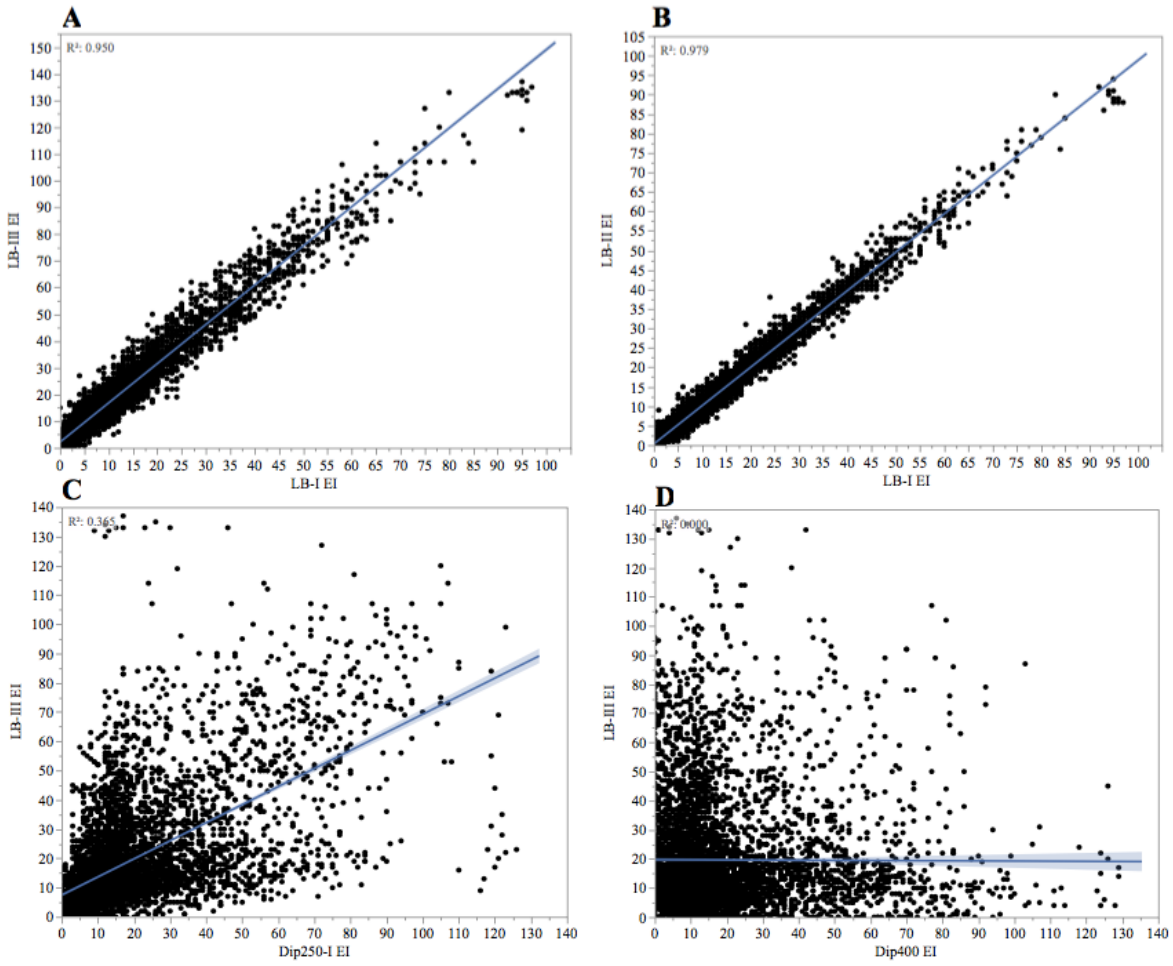


Fig S3.7. Tn-seq essentiality index (EI) correlation between LB and iron-restricted conditions. (A) EI correlation between two nonidentical transposon libraries, LB-III was grown in LB broth till mid-log phase but LB-I did not grow in LB broth (R^2: 0.95). (B) EI correlation between two identical transposon libraries, LB-II was grown in LB broth till mid-log phase but LB-I did not grow in LB broth (R^2: 0.98). (C) EI correlation between LB-III and an iron-restricted condition Dip250, 250 µM 2,2`-Dipyridyl ($R^2$: 0.37), cultures were grown till mid-log phase. (D) EI correlation between LB-III and an iron-restricted condition Dip400, 250 µM 2,2`-Dipyridyl (R^2: 0.00), cultures were grown till mid-log phase.

Selection conditions and summary of Tn-seq

We had two transposon Tn5 mutant libraries. The first library was named library-A which composed of 325,000 mutants. To identify essential genes with a high-confidence, the transposon library has to be hyper-saturated. Although 90% of ORFs in library-A had insertion, we constructed another transposon library to make sure that no gene left without insertions. The second library was combined with library-A and named library-AB which composed of 650,000 mutants. In library-AB, 92.6% of ORFs had insertions. Always, excluding the 5% of 5' and 10% of 3' of genes as disruption in the beginning of N-terminal and end of C-terminal may not affect function of the proteins. Library-A was inoculated into Luria-Bertani (LB) broth media supplemented with either 100 or 150 μM Dip, named Dip100 and Dip150, respectively, and LB free of Dip named LB-II. Then, we inoculated library-AB into LB media supplemented with either 250 or 400 μM Dip, named Dip250 and Dip400, respectively, and LB-III with no Dip. We also had a condition of library-A without growth, named LB-I. For LB-I, the inoculum was directly subjected to Tn-seq without inoculation to a growth medium (Fig. S3.1). The cultures were grown till the bacteria reached mid-log phase. LB-II and LB-III required ~5 h to reach mid-log phase, but Dip250 required 10 h. Since the growth of Dip400 was slow, we allowed the culture to grow for 24 h in order to its cell density to be close to other conditions (Table S3.1). The Dip400 culture was still in log-phase in the 24 h time of experiment (Fig. S3.2). As the concentration of Dip increased, the growth rate and maximum OD600 of cultures decreased (Fig S3.3). In this work, we used 273 million (M) sequence reads from Tn5 genomic junction in chromosome of S. Typhimurium for all conditions and 185 M sequence reads mapped to the genome. The number of unique insertions in chromosome were 125,499 in library-A and 193,728 in library-AB (Table S3.2). The high number of read counts and length of mapped reads allowed us to define gene essentiality with a high precision. Our initial goal in this study was to elucidate the conditionally essential genes that mediate survival of S.

Typhimurium in a gradient iron-restricted conditions using Tn-seq. However, we found mutants of scores of genes with increased fitness in iron-restricted conditions. Surprisingly, the majority of the genes with increased fitness are essential genes. This is contrary of all observations on essential genes because these genes should not tolerate disruptions or they can barely tolerate disruptions. However, we found significant insertions with many read counts in essential genes in iron-restricted conditions. Thus, we reasoned that the iron chelator ceased the death of cells that are defective in an essential gene and allowed these mutants to grow very slowly.

Essential genome of S. Typhimurium in iron-replete niches

To make sure our identified essential genes are critical for growth in LB broths and on LB agar plates, we used LB-I, LB-II, and LB-III for identification genes. Although Tn-seq data from these non-identical selection conditions are sufficient for essential genome analysis, robust Tn-seq analysis tools are required for essential gene identification. Several tools were developed for Tn-seq analysis such as Essentials, ARTIST, Tn-Seq Explorer, TRANSIT and each tool applies a distinctive algorithm in order to identify essential ORFs or regions in genome of bacteria. Our experience with Tn-seq analysis denotes that non of these tools are error-free. Eventually, we integrated analysis results of Tn-Seq Explorer and TRANSIT for the three LB conditions to find essential genes. Tn-Seq Explore calculates essentiality index (EI) based on number of insertions in genes, while TRANSIT applies extreme-value distribution to determine unusually long consecutive sequences lacking insertions. As the three conditions were analyzed by two tools, each gene had 6 calls. The gene was considered essential if it was essential in 5 out of 6 calls, with exception for a few genes (Fig. S3.4). As a result, we found 336 genes that are required for an aerobic robust growth of pathogenic S. Typhimurium 14028S in LB broths and on LB agar plates under laboratory conditions (Table S3.3). Because this is

the first report of essential genes in S. Typhimurium strain 14028S, we compared our essential gene list to the only reported essential genes in S. Typhimurium strain SL3261 by TraDIS approach. S. Typhimurium SL3261 is a derivative of SL1344 and an attenuated strain in mouse de to deletion of a few genes in the genome background. Interestingly, out of 336 genes in our essential list, 265 (80%) orthologous genes in S. Typhimurium SL3261 were also essential (Table S3.4). This is a very good match which obtained for bacteria across strains. The rest of non matched genes may be due to strain specific genes or variations in applied techniques. In addition, 205 (61%) of our identified essential genes have the same official gene symbol in the E. coli Keio collection. This implies the accuracy of our Tn-seq method and analysis for essential gene identification. KEGG pathway analysis recognized 306 out of 336 genes and categorized them into 23 essential pathways (Fig. S3.5).

Essential genome of S. Typhimurium in iron-restricted niches

Almost all of reported essential genes based on Tn-seq in bacteria are conducted in stress-free conditions. However, we were curious to assess the essential genome under iron-restricted conditions that mimic the niches Salmonella confronts in the host. We selected transposon libraries in gradient iron-restricted conditions and we applied the same analysis strategies that were used for iron-replete conditions to identify essential genes for Dip250, and Dip400 (Fig S3.5). Before essential gene identification, we calculated EI correlation between conditions. Albeit transposon libraries were not identical and LB-I was not grown for selection, but LB-II and LB-III, EI correlation between LB-III and LB-I was 0.95; LB-II and LB-I was 0.98 (Fig. S3.7 A and B). Remarkably, as the concentration of iron chelator elevated, the EI correlation between LB and Dip dropped. EI correlation between LB-III and Dip250 was 0.37 (Fig. S3.7 C) and the correlation became zero between LB-III and Dip400 (Fig. S3.7 D). That was a clue and an indicator that the mutants died or survived in LB were not the same in Dip

conditions. Surprisingly, we found only 215 essential genes of S. Typhimurium in iron-restricted conditions, Dip250 and Dip400 combined (Table S3.5). Thus, essential genome of S. Typhimurium in iron-restricted conditions decreased to 215 genes, 36% (121 genes) reduction compared to essential genome in iron-replete conditions. The number of insertions and reads in these 121 genes significantly elevated in iron-restricted conditions, consequently, the Tn-seq analysis algorithms did not consider them essential anymore. The average read counts in the 121 genes were 4.3 in LB-III whereas this elevated to 68 in Dip400. This is a clear evidence that the mutants of the 121 genes did not die and they grew slowly in iron-restricted conditions. In other words, chelation of iron in the media protected mutants of 121 genes and they grew either very slowly. The growth of these mutants are not viable but our Tn-seq approach was successfully identified them. This reduction of essential genes was very clear in Dip250 and Dip400, but not in Dip100 and Dip150. This is because iron was not limited enough by the applied concentrations of Dip100 and Dip150 and even their growth rates were not affected much as compared to LB conditions (Fig S3.3 and Table S3.3). The 215 genes were also essential under iron-restricted conditions in combined Dip250 and Dip400. The 121 genes are still essential for a robust growth but Tn-seq shows that their mutants can grow very slowly in iron-restricted conditions.

Electronic files

Table S3.3. Full list of *Salmonella* Typhimurium essential genes in iron-replete conditions identified by Tn-seq.

Table S3.4. The overlapped essential genes that identified by our Tn-seq method of *Salmonella* Typhimurium 14028S in iron-replete conditions and essential genes that identified by TraDIS method in *S*. Typhimurium SL3261.

Table S3.5. Full list of *Salmonella* Typhimurium essential genes in iron-restricted conditions identified by Tn-seq (ROS-independent essential genes).

Table S3.6. Full list of *Salmonella* Typhimurium essential genes in iron-restricted conditions identified by Tn-seq (ROS-dependent essential genes).

Table S3.7. Average sequencing read counts in essential genes of *Salmonella* Typhimurium in iron-replete and iron-restricted conditions identified by Tn-seq.

CHAPTER FOUR

Conclusion

In this work we utilized proteogenomics to elucidate the conditionally essential genes and proteins that are required for *Salmonella* Typhimurium resistance against H_2O_2. A robust, fast, and cost-effective Tn-seq method was developed. Validation of Tn-seq results with individual mutant assays indicated the accuracy of the identified genes in response to the two H_2O_2 concentrations. The coupled Tn-seq with targeted proteomics had a good agreement. Numerous genes were identified to have a role in resistance against H_2O_2. We found many genes that have not been associated for resistance to H_2O_2 previously. *Salmonella* employs multiple pathways to resist H_2O_2 and the most important ones are ROS detoxifying enzymes, amino acid biosynthesis (*aroK* and *aroB*), putative iron transporters (*ybbK*, *ybbL*, *ybbM*), iron homeostasis, Fe-S cluster repair, DNA repair, flagellar and DNA adenine methylase genes. Moreover, we identified the dynamics of conditionally essential genes that mediate *S.* Typhimurium survival in a gradient iron-restricted conditions. Surprisingly, our powerful Tn-seq approach indicated that when transposon mutant cultures treated with an iron chelator, the mutants of one-third of essential genome of *Salmonella* Typhimurium did not die and can grow slowly, however these mutants died in absences of the iron chelator. Based on this observation, we concluded that the iron chelator minimized ROS formation via downregulation of Fenton reaction, as a result, one-third of essential genes did grow likely because ROS is present in their death process. Thus, we divided essential genes into ROS-independent and ROS-dependent essential genes. The result is fitting to known model of common ROS-mediated antibiotic killing in bacteria and we further expand this model beyond antibiotic target genes. Strikingly, the targets of almost all antibiotics in clinical use are ROS-dependent essential genes. We propose that ROS-independent essential genes are better targets to develop new antimicrobials, as the cell die immediately following gene disruption. Altogether, this work

expands our knowledge about the genetic determinants that *Salmonella* operates to evade the host stressors.