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

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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.

59 **Importance**

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

Results and Discussion

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 S1). 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. S1, S2, S3). 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 S2). 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

149 between iron-replete and iron-restricted conditions.

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

151 We used rigorous analysis algorithms for essential gene identification (Fig. S4). As a result,
152 we identified 336 essential genes that are required for an aerobic growth of *S.* Typhimurium
153 14028S in LB broths and on LB agar plates (Table S3). We compared the essential genes in S.
154 Typhimurium 14028s to those in *S.* Typhimurium SL3261 identified by TraDIS approach (13).
155 Interestingly, out of 336 genes in our essential list, 265 (80%) orthologous genes in *S.*
156 Typhimurium 14028s were also essential in S. Typhimurium SL3261 (Table S4). This is a very
157 significant overlap considering variations in genetic backgrounds of the two strains. Further,
158 KEGG pathway analysis recognized 306 out of 336 genes and categorized them into 23
159 essential pathways (Fig. S5).

160 We also analyzed the essential genes from the Tn-seq data obtained from iron-restricted
161 conditions using the same rigorous algorithms. Surprisingly, the number of essential genes
162 under iron-restricted conditions decreased to 215 genes, which indicated that 121 genes (36%)
163 of the 336 essential genes are not considered essential under iron-restriction conditions (Table
164 S5). The number of insertions and reads in these 121 genes significantly increased under iron-
165 restricted conditions: the average read counts in the 121 genes were 4.3 in LB-III whereas this
166 elevated to 68 in Dip400 (Table S6). This is a clear evidence that the mutants of the 121 genes
167 not only did not die but also multiplied slowly in iron-restricted conditions. In other words,
168 chelation of iron in the media allowed the mutants of these 121 genes to escape immediate
169 killing and to multiply.

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

171 When transposon mutants grow in liquid media, the rapidly growing mutants out compete the
172 slowly growing ones (18, 19). As a result, Tn-seq sequencing reads cannot be obtained for

173 these slowly growing mutants and the genes disrupted in these mutants would be considered
174 important for fitness under that condition. To check how significant this phenomenon in our
175 Tn-seq and whether the sequencing read counts we obtained from Dip conditions resulted from
176 minor competitions of mutants because the growth rate in Dip400 decreased 26.4% compared
177 to LB (Fig. S3). We utilized LB-I Tn-seq data. Library was recovered on agar plates following
178 mutagenesis and colonies had enough space on the plates in order to not compete for nutrients.
179 Although the trend showed increase of insertions in genes in LB-I compared to broth culture,
180 LB-II, the number of essential genes to be called non essential were slight. The average read
181 counts in the 336 essential genes (always, excluding 5% 5' end and 10% 3' end) in LB-I and
182 LB-II were 5.1 and 2.1, respectively, while this number was 30.6 in Dip400 (Table S7).
183 Therefore, this emphasizes that increasing of insertions and read counts in many essential genes
184 in Dip conditions are not result of reduced competitions and it is likely having a connection
185 with iron.

186 We next asked whether increase of insertions and read counts in essential genes of Dip
187 conditions were due to a bias in the Tn-seq approach. We conducted analysis for identification
188 of essential genes without data normalization and there were differences in read counts in Tn-
189 seq conditions (Table S2). For instance, the total read counts in ORFs of LB-III was ~30
190 millions (M) versus ~16 M in Dip400, excluding intergenic regions. Two mutants,
191 *STM14_2422* (*umuC*) and *STM14_2428*, consumed 8.7% of reads in LB-III and 27% in
192 Dip400. Consequently, on average an insertion in LB-III had chance to get 227 reads while in
193 Dip400 the chance was dropped to only 100 reads for an insertion (Table S8). This indicates
194 that the bias in read counts was in favor to not see insertions and read counts in essential genes
195 in iron-restricted conditions. Even though with this bias in read counts which partially produced
196 by these two mutants, the read counts in the 121 genes were higher in Dip compared to LB.
197 This is a strong evidence that these 121 mutants, specifically 33 genes, are genuine slowly

198 growing mutants in iron-restricted conditions.

199 **Fitness of slowly growing mutants of essential genes increased**

200 We next measured fitness of the 121 genes in Dip250 and Dip400 (outputs) and LB-III (input).
201 Strikingly, fitness of 97 out of 121 genes (78%) were increased in Dip400, the rest of the 121
202 genes were either had increased fitness in Dip250-I, or Dip250-II. Further analysis indicated
203 that number of essential genes with increased fitness in presence of Dip were 33 including
204 *gyrA*, *gyrB*, and *ileS*, p values < 0.05 (Table 1). *gyrA*, *gyrB*, and *ileS* were not in the list of 121
205 genes, whereas fitness of their mutants increased significantly. This is another strong evidence
206 that the genes with positive fitness in iron-restricted conditions are slowly growing mutants
207 and the iron chelator cased their death.

208 **Essential genes are not condition-specific**

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

220 **ROS-dependent and ROS-independent essential genes**

221 We finally came to a conclusion that iron-restriction allowed the growth of the mutants of

222 essential genes and ceased or slowed down the killing process. This hypothesis is related to the
223 ROS-mediated common killing mechanisms of bactericidal antibiotics. Since its first proposal
224 by Kohanski et al. (5), this hypothesis has been substantiated by numerous studies using
225 different bacterial species and bactericidal antibiotics. Traditionally, the mechanisms of
226 antibiotic action have been studied largely in terms of antibiotic-target interactions. However,
227 numerous researches supporting the ROS-mediated killing mechanism and have shown that
228 the interaction of antibiotic-target leads to production of ROS, contributing to the killing
229 activity mediated by direct blocking of the basic pathways for living cells. We believe that
230 similar processes occurred by disruption of genes of essential proteins with transposons. Thus,
231 mutants of essential genes that grow slowly in iron-restricted conditions are ROS-dependent
232 essential genes which are 121 genes (Table S6) and mutants of essential genes that do not grow
233 in iron-restricted and iron-replete conditions are ROS-independent essential genes which are
234 215 genes (Fig. 1, Table S5).

235 Until now this proposed mechanism has been discussed with focus on the genes that have been
236 exploited as targets of a limited number of bactericidal antibiotics. Interestingly, our Tn-seq
237 data show that the majority of targets of the bactericidal antibiotics are ROS-dependent
238 essential genes (Fig. 1), which implies that lethal effect of the knockout of the antibiotic target
239 genes were reduced to varying extent by restriction of available iron in the media. Our Tn-seq
240 data show that this ROS-mediated killing mechanism is linked to about one-third of the
241 essential genes, far beyond a limited number of genes encoding targets of bactericidal
242 antibiotics, thereby expanding the "common" nature of the ROS-mediated lethal pathway as a
243 universal mechanism connected to a broad range of basic essential pathways for life. By our
244 definition, based on Tn-seq, ROS-independent essential genes, 215 genes, are required for a
245 robust growth and viability, the cells die upon disruption of the genes, chelation of iron can not
246 rescue their mutants from death, and their average read counts are 9.6 in Dip400. While ROS-

247 dependent essential genes, 121 genes, are required for a robust growth and viability, the cells
248 do not die directly upon disruption of the genes, chelation of iron can cease the death process
249 of their mutants, they grow very slowly in iron-restricted conditions but not in iron-replete, and
250 their average read counts are 67.9 in Dip400 (Fig. 2). Further, we show that these ROS-
251 dependent essential genes are part of 9 essential pathways (Fig. 3).

252 **Fitting ROS-dependent essential genes in ROS-mediated antibiotic killing model**

253 ROS-mediated antibiotic killing model has a few components: (i) antibiotic-target interactions
254 (disruption or blockage of an essential pathway), (ii) induce NADH oxidation via the electron
255 transport chain which depends on TCA cycle, (iii) induce superoxide formation via the electron
256 transport chain, (iv) superoxide damages Fe-S clusters and the released ferrous iron fuels
257 Fenton reaction, (v) the consequence of Fenton reaction is hydroxyl radical formation which
258 leads to damage of biomolecules and ultimately cell death. Juxtaposing our work to this model,
259 we impaired the essential gene or pathway by disrupting the gene with Tn5 transposon, no
260 antimicrobial interference. Regarding the role of TCA cycle in this model, Kohanski et al., (5)
261 deleted the genes in TCA cycle that produce NADH and they found that *E. coli* lacking either
262 *acnB* or *icdA* had increased survival following antibiotic treatment, however, other genes in
263 TCA cycle pathway that follow *acnB* and *icdA* such as *sucB* and *mdh* did not have protective
264 effect following antibiotic treatment because NADH already formed in the pathway by *acnB*
265 and *icdA*. Astonishingly, we found that fitness of *acnB* and *icdA* increased in iron-restricted
266 conditions (Table 2); *S.* Typhimurium lacking *acnB* or *icdA* can grow better in iron-restricted
267 conditions, whereas the fitness of other mutants in TCA cycle did not change in iron-restricted
268 conditions. This emphasizes the role of NADH and TCA cycle in ROS formation and bacterial
269 cell death. Evidence for the contribution of TCA cycle in ROS-mediated killing has increased.
270 A dysfunctional TCA cycle in *Staphylococcus epidermidis* enhanced survival following β-
271 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. 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 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. S6). 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*

297 is participated in Fe-S cluster biosynthesis in *S. Typhimurium* in Dip250 and Dip400.
298 Collectively, this demonstrates the role of Fe-S clusters and other genes in homeostasis of iron
299 that directly or indirectly fuel Fenton reaction.

300 The fitness of the subunits of DNA polymerase V, *umuDC*, increased in iron-restricted
301 conditions which is an indicator that *S*. Typhimurium lacking umuDC was grown better in iron-
302 restricted conditions. In *E. coli*, a mutant strain lacking dnaE911, DdinB, and DumuDC is more
303 resistant to killing by bactericidal antibiotics than wild-type; DNA polymerase III, IV, and V
304 contribute to ampicillin-mediated cell death. Particularly, the generated ROS (hydroxyl
305 radicals) following antibiotic treatment oxidizes guanine nucleotide pool to a mutagenic 8-oxo-
306 deoxyguanosine (8-oxo-guanine) which results in lethal outcomes because incorporation of 8-
307 oxo-guanine into DNA causes double-strand breaks (28). The essential genes *dnaEX* which
308 encode subunits of DNA polymerase III and the conditionally essential genes *umuDC* which
309 encode subunits of DNA polymerase V had increased fitness based on Tn-seq in iron-restricted
310 conditions. The uncharacterized ORF *STM14_2428* is a neighbor of *STM14_2422* (*umuC*) had
311 also increased fitness (Table 2), but it is unclear how *STM14_2428* deletion is in favor *S.*
312 Typhimurium growth in iron-restricted conditions. Interestingly, two important conditionally
313 essential genes, *guaB* and *purA* had increased fitness. These two genes catalyze the first step
314 in the de novo synthesis of guanine and adenine from inosine 5'-phosphate (IMP). These genes
315 may have a connection with 8-oxo-guanine, however we are devoid of evidence to support the
316 role of *guaB* and *purA* in ROS pathways. Tn-seq shows that *S*. Typhimurium was grown better
317 in iron-restricted conditions when *guaB* or *purA* deleted. These findings emphasize the role of
318 DNA polymerases in ROS-mediated killing as deleting of these genes results of a better
319 bacterial growth in iron restricted conditions.

We have several other genes that identified by Tn-seq with either increased or decreased fitness (Table 2 and Fig. 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.

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. 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 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.

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 contribute 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 expanded 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.

Materials and Methods

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).

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.

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.

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 Reaerch). Then, a linear PCR extension was performed using Tn5-DPO (5'-AAGCTTGCATGCCTGCAGGTIIIIICTAGAGGATC-

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, AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGA TCTNNNNAG-BARCOD-CCTAGGCGGCCTTAATTAAAGATGTGTATAAGAG and P7-16G primer, CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTGGGGGGGGGGGGGGG. 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 Reaerch). 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.

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.

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 $\leq$ 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.

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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Author Contributions

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

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Author Contributions

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

709 Table S3. Full list of *Salmonella* Typhimurium essential genes in iron-replete (LB) conditions
710 identified by Tn-seq.
711 Table S4. The overlapped essential genes that identified by our Tn-seq method of *Salmonella*
712 Typhimurium 14028S in iron-replete (LB) conditions and essential genes that identified by
713 TraDIS method in *S.* Typhimurium SL3261.
714 Table S5. Full list of *Salmonella* Typhimurium essential genes in iron-restricted conditions
715 identified by Tn-seq (ROS-independent essential genes). Iron chelator 250 and 400 μM 2,2`-
716 Dipyridyl were used.
717 Table S6. Full list of *Salmonella* Typhimurium essential genes in iron-restricted conditions
718 identified by Tn-seq (ROS-dependent essential genes). Iron chelator 250 and 400 μM 2,2`-
719 Dipyridyl were used.
720 Table S7. Average sequencing read counts in essential genes of *Salmonella* Typhimurium in
721 iron-replete (LB) and iron-restricted (Dip400) conditions identified by Tn-seq.
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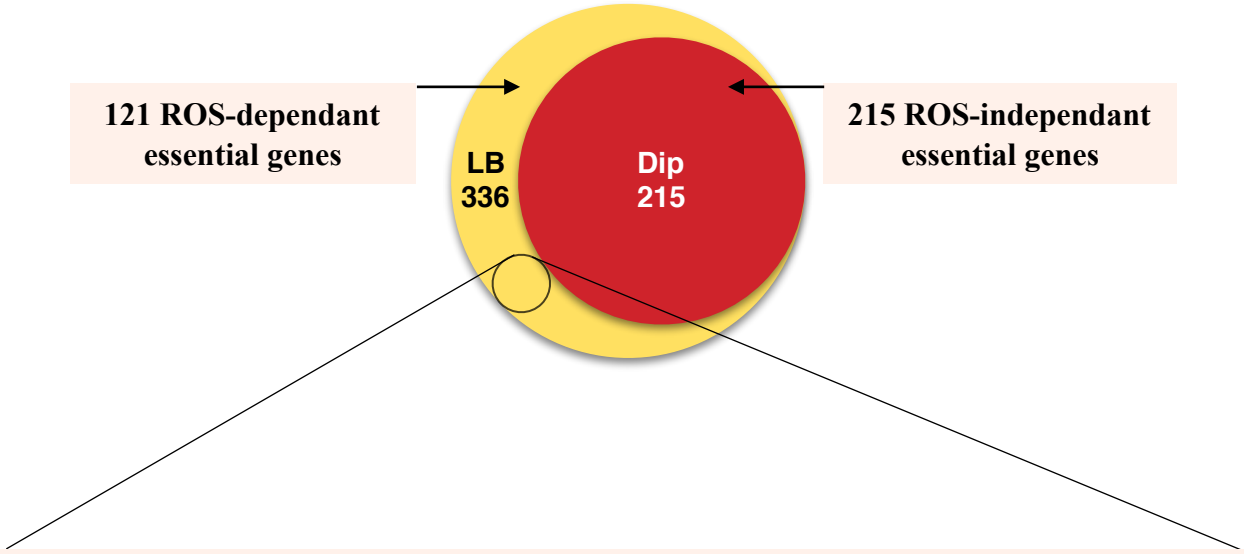
Table 1. Essential genes have increased insertions and read counts in iron-restricted conditions
(LB: iron-replete, Dip: iron-restricted, I: insertion, R: read count, fitness: Log2 fold change)

		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
Transcription	***rpoC***	1	1	9	186	10	150	7.23	0
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 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, an indicator of growth of the mutant. The fitness of these 33 genes increased in Dip400, 400 µM of iron chelator. Gene fitness is Log2 fold change of sequence reads, Dip400 vs LB. Read

Essential genome of *S.* Typhimurium in iron-replete (LB) and iron-restricted (Dip)



Of the 121 ROS-dependant essential genes, fitness of 33 mutants increased significantly in iron-restricted conditions (Dip400), $p < 0.05$, and proteins of 11 genes are well known antibiotic targets

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 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. Read counts increase is an indicator mutant growth. 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.

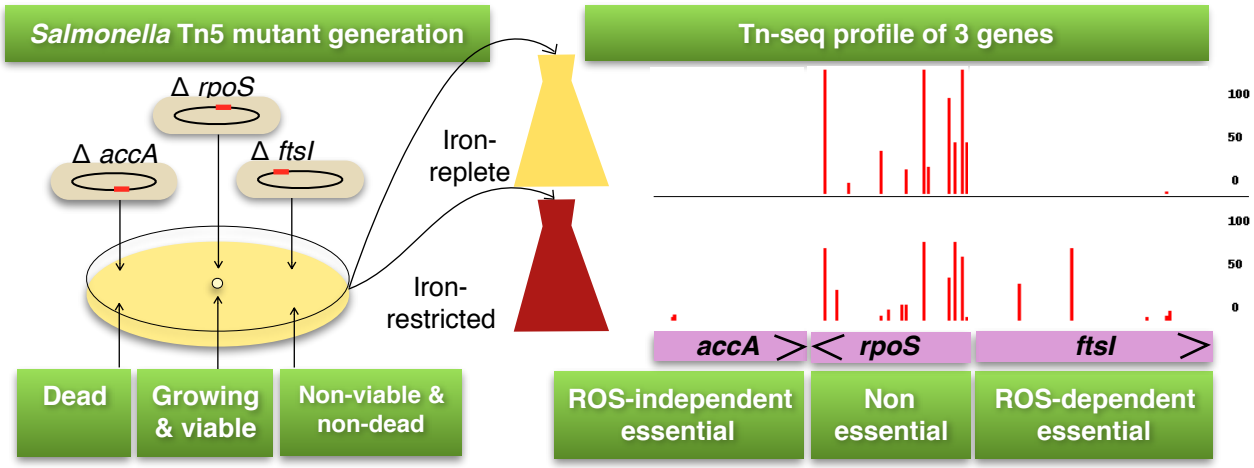


Fig 2. Identification of ROS-dependent essential genes by Tn-seq. *Salmonella* Typhimurium Tn5 mutants generated and the library inoculated to iron-replete media (LB broth) and iron-restricted media (LB supplemented with an iron chelator 2,2`-Dipyridyl). The cultures were grown till mid-log phase and 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 (0-100) are read counts and each red line represent a unique insertion.

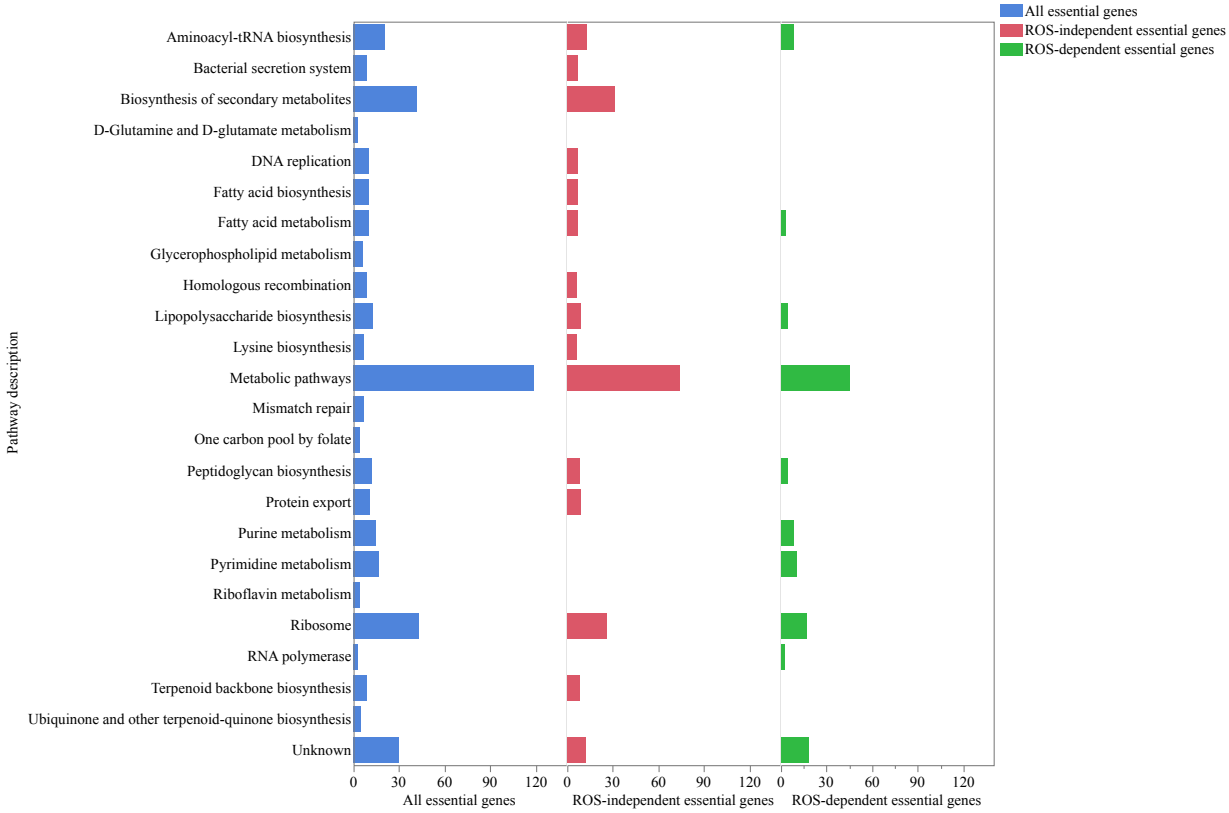


Fig 3. ROS-dependent essential pathways in *Salmonella* Typhimurium identified by Tn-seq. Transposon libraries were grown in iron-replete and iron restricted conditions. Essential genes identified for both conditions. The genes that were essential in iron-replete condition but not in iron-restricted conditions were considered ROS-dependent. KEGG pathway analysis was used for pathway description.

Table 2. 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
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 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 of 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 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.

	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 S1: 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. OD_{600} 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.

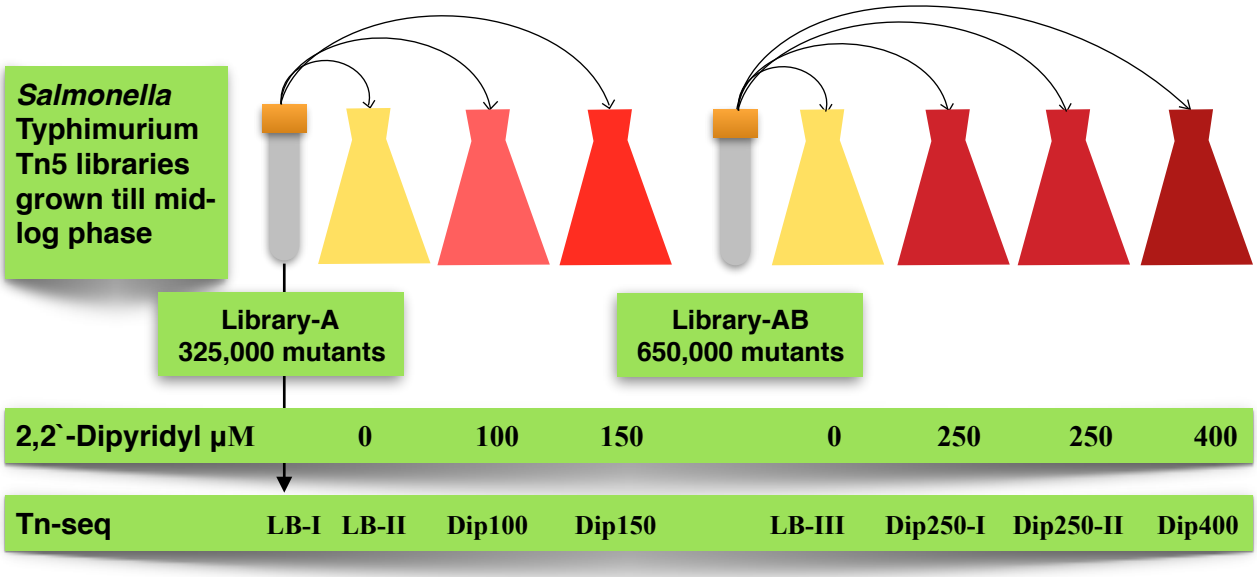


Fig S1: Schematic representation of study design. Transposon library-A inoculated to LB broth (LB-II) or the LB contained either 100 µM iron chelator Dip (Dip100) or 150 µM Dip (Dip150). LB-I was library-A that subjected to Tn-seq without growth. Transposon library-B inoculated to

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.

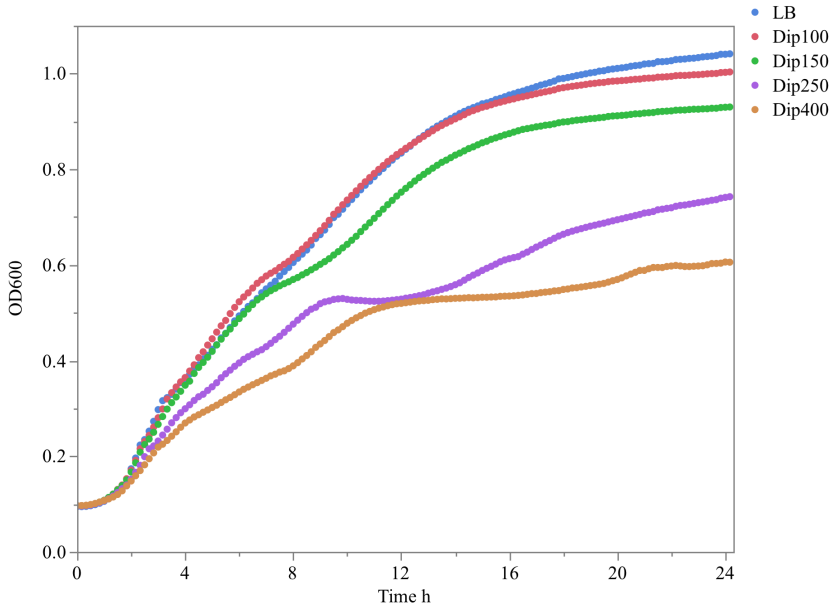


Fig S2: Effect of 2,2`-Dipyridyl (Dip) on *S.* Typhimurium growth. An overnight culture diluted 1:200 in LB broth supplemented with 100 µM Dip (Dip150), 150 µM Dip (Dip150), 250 µM Dip (Dip250), 400 µM Dip (Dip400), or Dip free (LB). The cultures were added to a 96-well plate and directly incubated at 37^{o}C in a plate reader, reading OD_{600} every 10 minuets.

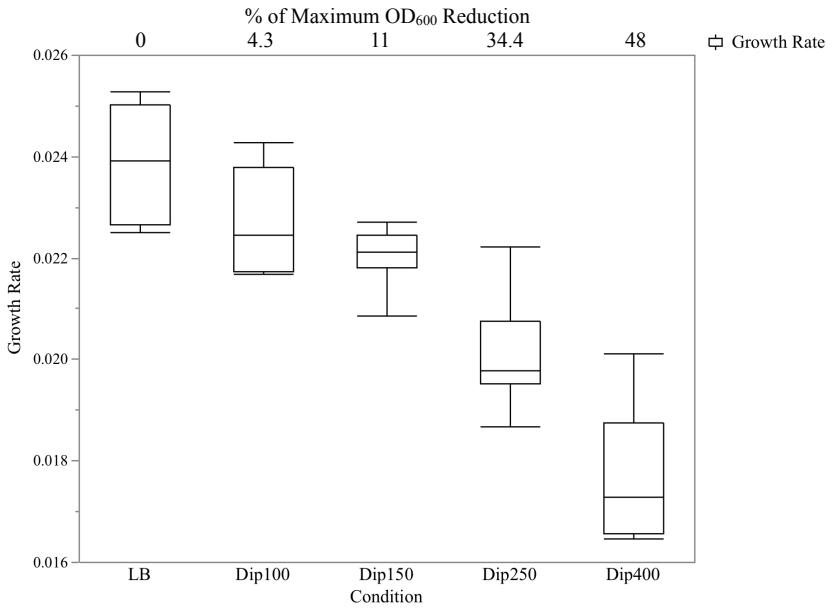
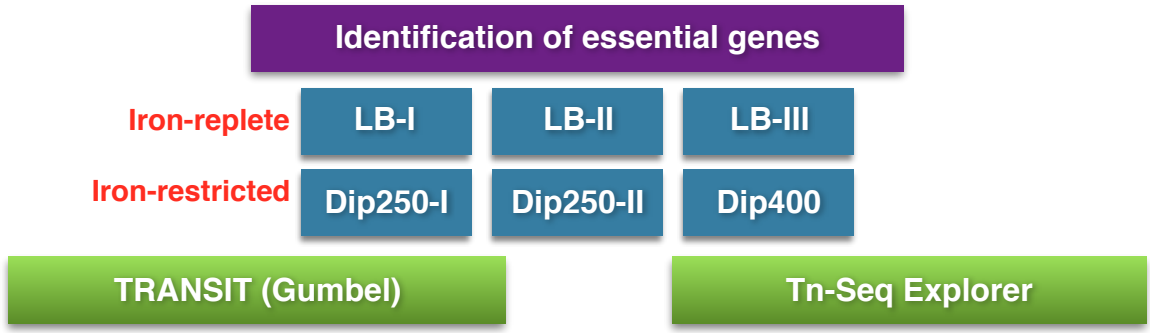


Fig S3. Effect of 2,2`-Dipyridyl (Dip) on *S.* Typhimurium growth rate and cell density. An overnight culture diluted 1:200 in LB broth supplemented with 100 µM Dip (Dip150), 150 µM Dip (Dip150), 250 µM Dip (Dip250), 400 µM Dip (Dip400), or Dip free (LB). The cultures were added to a 96-well plate and directly incubated at 37^{o}C in a plate reader, reading OD_{600} every 10 minuets. The maximum OD_{600} 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 S2: 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 S4: 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 analysis was 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 S5. 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.

Table S8: Bias in Tn-seq sequencing reads

	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 S8. 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).

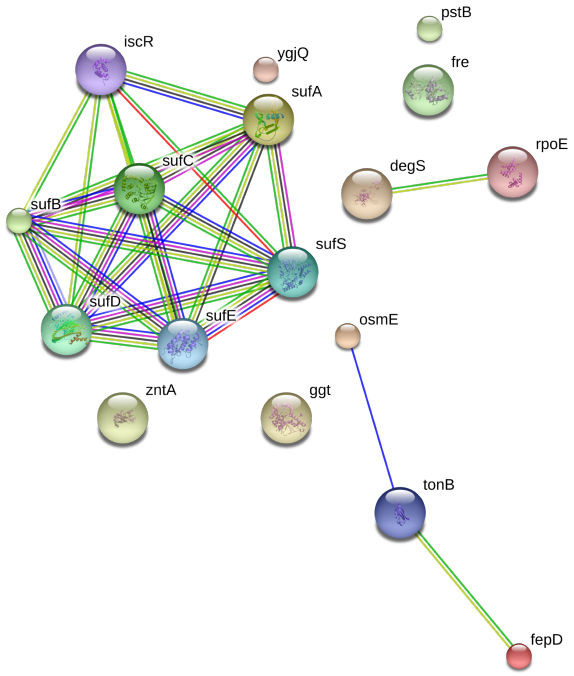


Fig S6. Protein-protein interactions of conditionally essential genes that mediate *Salmonella* Typhimurium survival in iron-restricted conditions. Transposon mutant libraries inoculated to LB (free of iron chelator Dip) and LB supplemented with various concentrations of Dip. The cultures were grown till mid-log phase. The gene fitness measured by Tn-seq. The interaction analysis conducted by using default settings of STRING.

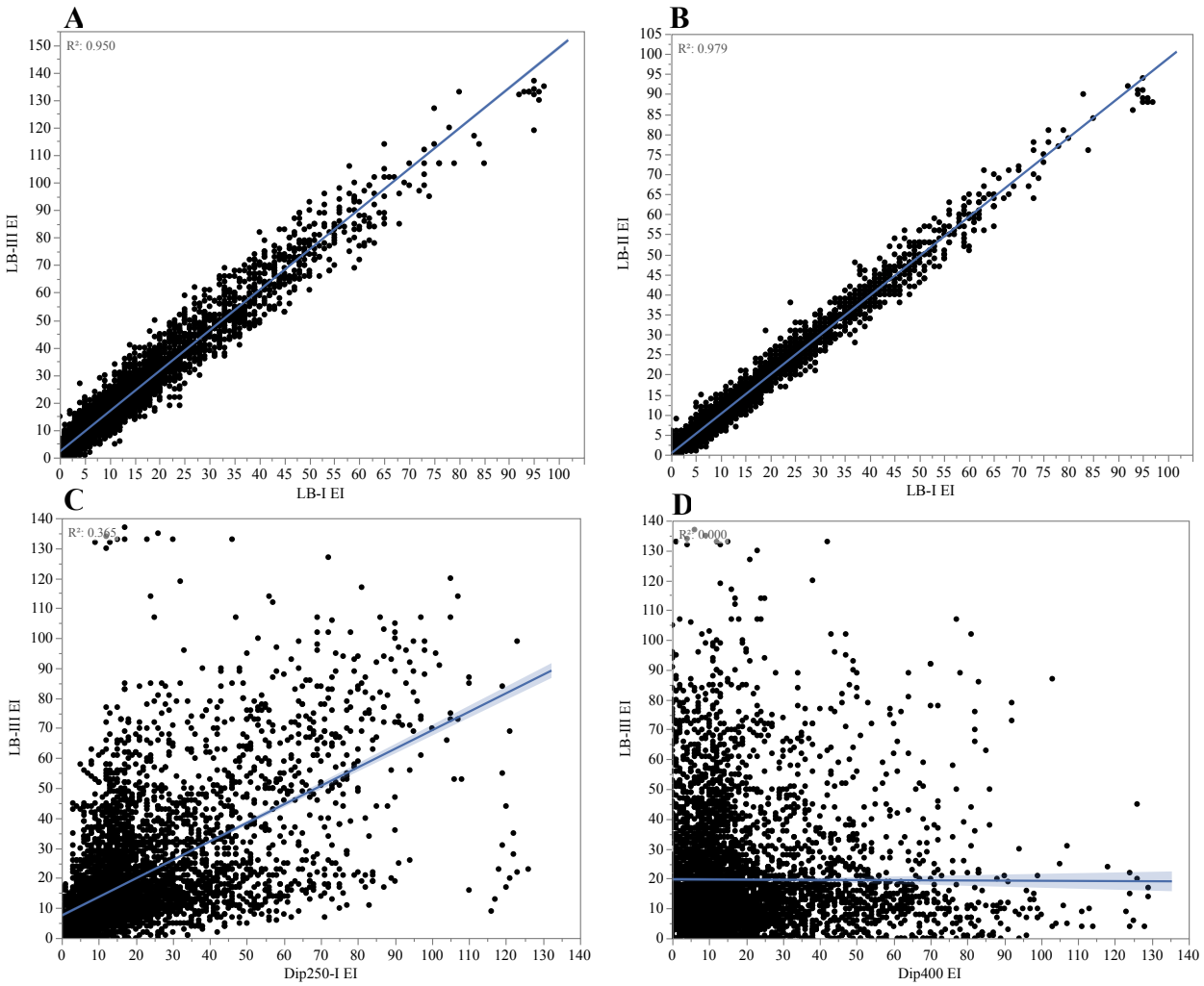


Fig S7. Tn-seq essentiality index (EI) correlation between LB and iron-restricted conditions. (A) EI correlation between two nonidentical transposon libraries, LB-III was grown in LB broth till mid-log phase but LB-I did not grow in LB broth (R^2: 0.95). (B) EI correlation between two identical transposon 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. S1). 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 S1). The Dip400 culture was still in log-phase in the 24 h time of experiment (Fig. S2). As the concentration of Dip increased, the growth rate and maximum OD600 of cultures decreased (Fig S3). 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

S2). 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. S4). 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). 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 S4). 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. S5).

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 S5). 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. S7 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. S7 C) and the correlation became zero between LB-III and Dip400 (Fig. S7 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 S5). 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 and Table S3). 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.