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# Global transcriptomic analyses of *Salmonella enterica* in Iron-depleted and Iron-rich growth conditions

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## Abstract

**Background:** *Salmonella enterica* possess several iron acquisition systems, encoded on the chromosome and plasmids. Recently, we demonstrated that incompatibility group (Inc) FIB plasmid-encoded iron acquisition systems (Sit and aerobactin) likely play an important role in persistence of *Salmonella* in human intestinal epithelial cells (Caco-2). In this study, we sought to determine global transcriptome analyses of *S. enterica* in iron-rich (IR) and iron-depleted (ID) growth conditions.

**Results:** The number of differentially-expressed genes were substantially higher for recipient (SE819) ( $n = 966$ ) and transconjugant (TC) ( $n = 945$ ) compared to the wild type (WT) (SE163A) ( $n = 110$ ) strain in ID as compared to IR growth conditions. Several virulence-associated factors including T3SS, flagellin, cold-shock protein (*cspE*), and regulatory genes were upregulated in TC in ID compared to IR conditions. Whereas, IS1 and *acrR/tetR* transposases located on the IncFIB plasmid, ferritin and several regulatory genes were downregulated in TC in ID conditions. Enterobactin transporter (*entS*), iron ABC transporter (*fepCD*), colicin transporter, IncFIB-encoded enolase, cyclic di-GMP regulator (*cdgR*) and other regulatory genes of the WT strain were upregulated in ID compared to IR conditions. Conversely, ferritin, ferrous iron transport protein A (*feoA*), IncFIB-encoded IS1 and *acrR/tetR* transposases and ArtA toxin of WT were downregulated in ID conditions. SDS-PAGE coupled with LC-MS/MS analyses revealed that siderophore receptor proteins such as chromosomally-encoded IroN and, IncFIB-encoded *lutA* were upregulated in WT and TC in ID growth conditions. Both chromosome and IncFIB plasmid-encoded SitA was overexpressed in WT, but not in TC or recipient in ID conditions. Increased expression of flagellin was detected in recipient and TC, but not in WT in ID conditions.

**Conclusion:** Iron concentrations in growth media influenced differential gene expressions both at transcriptional and translational levels, including genes encoded on the IncFIB plasmid. Limited iron availability within the host may promote pathogenic *Salmonella* to differentially express subsets of genes encoded by chromosome and/or plasmids, facilitating establishment of successful infection.

**Keywords:** *Salmonella*, Iron acquisition systems, Transcriptomic analysis, Iron-depleted growth conditions, Iron-rich growth conditions

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## Background

*Salmonella enterica* is one of the major foodborne pathogens in the United States [1, 2], often associated with multistate outbreaks linked to contaminated foods and food products or pet animals such as turtle [3] and hedgehogs [4]. *Salmonella* can cause a wide range of human infections, from mild gastroenteritis to invasive diseases [5]. Over 2600 *Salmonella* serovars have been identified, largely differing in host ranges and their ability to cause human infections [5]. *Salmonella enterica* serovars such as Enteritidis, Typhimurium, Newport, and Heidelberg can colonize intestines of a broad range of hosts including food producing animals and humans [6]. On the other hand, some *Salmonella* serovars are host-restricted such as Typhi, Paratyphi, Gallinarum, Choleraesuis, Abortusovis and Dublin, which can only cause infections in one or few hosts [7]. Nonetheless, genetic factors that contribute to broader host range and increased ability to cause invasive form of disease to different *Salmonella* serovars are still largely unknown.

*Salmonella* possess arrays of genes that aid in invasion, replication, and persistence inside the host cells [8]. Type III secretion systems (T3SS) are among the major factors that play roles in invasion and persistence in the host cells [9–11]. *Salmonella* pathogenicity islands (SPIs) encode virulence factors, including T3SS, that are required during infections of host cells [12]. Genomes of *Salmonella* acquire SPIs through different evolutionary processes via horizontal gene transfer (HGT). To date, 21 SPIs have been identified in *S. Typhimurium*, among which SPI-1 and SPI-2 are well studied [9–11, 13]. SPI-1 contains genes, which are essential for the early infection process to host cells, while SPI-2 contains genes that contribute to late infection processes of survival and replication inside host cells [14]. SPI-1 and SPI-2 harbor T3SS-1 and T3SS-2, respectively, and regulate expression of virulence genes including secretion of T3SS effector proteins in a highly regulated manner to establish infection in the host [15–17]. Besides chromosomally-mediated virulence factors, we and others have demonstrated that virulence plasmid-encoded factors such as iron acquisition systems located on incompatibility group (Inc) FIB plasmids [18], VirB/D4 type 4 secretion systems encoded on the IncX4 plasmid, [19] and the *spv* operon located on multiple virulence plasmids [20] likely contribute to increased virulence of *Salmonella* during infection of host cells. However, the precise role of these virulence-associated plasmid factors of *Salmonella* in infection process remains to be determined.

*Salmonella* serovars encounter different challenging environments during host-pathogen interactions, including iron-limited conditions inside the host cells. Iron is not only an essential growth factor for many pathogenic bacteria [21], but also serves as a signaling element that

regulates various genes, including virulence associated genes [22]. The master regulator, Fur (ferric uptake regulator), senses iron availability and controls gene expression as necessary, in response to physiological conditions [23–25]. Previous studies have shown that a Fur mutant attenuated the virulence and pathogenesis observed in vivo models for several pathogens [24, 26–29].

For most bacteria, iron acquisition is one of the key factors that determine their ability to survive in a host [30]. Bacteria that do not possess iron acquisition capability, may be removed by the host defense mechanisms [30]. Some of the common host defense mechanisms are: i) to limit iron availability to pathogen by forming iron-host protein complexes. The host produces a specific class of the glycoprotein transferrin, such as serotransferrin and lactoferrin, which have iron-binding capability [30–32]. ii) to respond directly to invasive pathogens by altering the status of internal host iron concentrations. Host cells, such as macrophages produce Nramp (natural resistance-associated macrophage protein) proteins that control internal iron status in response to infection [30]; and iii) to impact host defense cells, such as macrophages which require iron for production of toxic reactive oxygen intermediates (ROI) and reactive nitrogen intermediate (RNI) to stimulate oxidative damage to eliminate pathogens [21].

*Salmonella* have evolved with different iron acquisition systems, encoded on the chromosome and plasmids, which facilitate them to chelate iron from the host to establish successful infection [22, 33]. A previous study showed that the *sitABCD* iron transporter operon located on the SPI-1, is induced under iron-depleted conditions [34]. Additionally, they demonstrated that genes of the *sitABCD* operon are repressed by Fur [34]. The same study showed that the SitABCD (Sit) system was increasingly expressed after invasion of the intestinal epithelium and a Sit null mutant was attenuated in animal experiment [34]. These data indicate that chromosome-encoded Sit iron transport system contributed to virulence of *S. Typhimurium*. Incompatibility (Inc) group FIB plasmids can also encode the Sit and the aerobactin iron acquisition (*iucABCD-iutA*) systems [18, 35]. Mechanisms of regulation of plasmid-mediated Sit and aerobactin iron acquisition systems have yet to be determined.

*Salmonella* possess several regulatory systems that facilitate control of gene expression in a highly regulated manner to survive in different environmental niches [36–38]. A previous study investigated transcriptomic analyses of 22 distinct infection-relevant environment conditions to mimic infection in host using RNA-Seq [38]. They showed that different in vitro conditions stimulate characteristic transcriptional signatures genes, in which 22 conditions influenced transcriptions of 86%

of total genes of *S. Typhimurium* [38]. Additionally, Hautefort et al. (2008) demonstrated that *S. Typhimurium* undergoes time-dependent transcriptional adaptation resulting simultaneous expression of three T3SS systems during infection of epithelial cells [39].

In a recent study, we demonstrated that IncFIB plasmid-encoded iron acquisition systems (Sit and aerobactin) likely play an important role in persistence of *Salmonella* in human intestinal epithelial cells [18]. In this study, we sought to determine the global iron regulated transcriptomes and proteome levels of selected protein bands of *S. enterica*. We identified differential expression of several genes located in the chromosome and on IncFIB plasmids at the transcriptional level using RNA-Seq. Selected differentially-expressed proteins were also determined by nano-liquid chromatography-tandem mass spectrometry (LC-MS/MS). The finding of the study will be useful for better understanding the influence of iron on the transcriptomes and proteomes of *Salmonella*.

## Methods

### Bacterial strains and growth medium

*Salmonella enterica* recipient SE819 and wild type SE163A strains isolated from food sources were sequenced in our laboratory [40]. A transconjugant (SE819::IncFIB) was developed in our previous study [18]. Ferric chloride (100  $\mu$ M) and 2,2'-bipyridyl (200  $\mu$ M) (Sigma-Aldrich, St. Louis, MO) were added to LB broth to prepare iron-rich (IR) and iron-depleted (ID) growth media, respectively as described in previous studies [41, 42].

### RNA isolation and quality assessments

Bacterial strains were sub-cultured on sheep's blood agar plates (Remel, Lenexa, KS) and single colonies of wild type, recipient, and transconjugant (SE819::IncFIB) strains were picked and grown in IR and ID LB broth overnight at 37 °C with shaking. The next day, overnight cultures were diluted at ratio of 1:20 in to corresponding fresh IR and ID LB medium. RNA was isolated from approximately  $10^9$  cells collected at mid-logarithmic phase from two biological replicates of each strain with the Ribopure Bacterial RNA Isolation Kit (Ambion, Invitrogen, Carlsbad, CA), following manufacturer's instructions. Genomic DNA was removed by treatment with DNase I (Ambion, Invitrogen, Carlsbad, CA).

Total RNA concentration was measured with Qubit fluorometer using RNA BR Assay kit (ThermoFisher Scientific, Waltham, MA), and the quality of RNA was examined by determining RNA integrity number (RIN) using Agilent Bioanalyzer 2100 with RNA 6000 nano kit (Agilent, Santa Clara, CA). Samples with RIN values  $\geq 7$  were subjected to library preparation for RNA-seq.

### Library preparation and RNA sequencing

rRNA was depleted from 5  $\mu$ g of total RNA from each sample using the ribo-zero rRNA removal kit (Illumina, San Diego, CA) according to instruction manual. Briefly, for each reaction, in the first step 225  $\mu$ L magnetic beads were washed. Then, ribo-zero removal solution was added to each RNA sample in order to hybridized probe to rRNA present in the samples. Washed beads and probe-hybridized samples were mixed together to remove rRNA from the samples. rRNA depleted samples were cleaned up by standard ethanol precipitation, and the quality of rRNA-depleted samples was assessed using the Agilent Bioanalyzer 2100 as described above.

RNA-Seq libraries were prepared by Truseq Stranded mRNA kit (Illumina, San Diego, CA) using 160 ng of each rRNA depleted sample. Briefly, first and second strand cDNA was synthesized and adenylated 3' ends of the blunt fragments to prevent them from ligating to one another during the adaptor ligation step. After that adaptors were ligated and selective DNA fragments with adaptors were enriched using PCR performed with a PCR primer cocktail that anneals to the ends of the adapters. The quality of cDNA was examined using Bioanalyzer with Agilent DNA 1000 kit (Agilent, Santa Clara, CA). After validation, libraries were quantified using KAPA NGS library quantification kit (Kapa Biosystem, Wilmington, MA) by quantitative reverse transcription-PCR (qRT-PCR) using SYBR green assays (Bio-Rad, Hercules, CA). RNA-Seq libraries were normalized, pooled, and sequenced by Illumina NextSeq platform using 2  $\times$  75 pair-end (PE) format. RNA sequencing was performed at Center for Food Safety and Applied Nutrition, FDA, College Park, MD.

FASTQ files generated from NextSeq were imported into CLC Genomics Work Bench version 9.0 (Qiagen, Redwood City, CA). In order to accommodate genes located on the IncFIB plasmid, sequence reads were mapped to the genome sequences of *S. Typhimurium* LT2 (lacks IncFIB plasmid) and the WT SE163A. For transcriptome analyses, a combined gene track from annotated draft genome of SE163A and the complete genome of LT2 were established using CLC Genomics Workbench. The gene name indicated by AY603\_XXXXX is mapped from SE 163A and the rest of the genes are mapped from LT2. Transcripts per million (TPM) was calculated for individual genes using CLC Genomics Workbench. Comparison of differential gene expression between two groups was conducted and fold-changes were determined by 'Exact Test' [43] and EdgeR [44], a bioinformatic tool for empirical analysis of differential gene expressions (designated as experimental fold-changes). Additionally, TPM of each gene was normalized with geometric mean TPM value of *gmk* and *adk* and fold-changes were calculated

from normalized values (designated as normalized fold-changes). Normalized value with  $\geq 4$ -fold differences of transcript abundances between ID and IR growth conditions were considered to demonstrate the presence of differential changes in gene expression (upregulation or downregulation) among the *Salmonella* strains compared. Total of 12 RNA sample libraries, representing two biological replicates for each strain in two different conditions (ID and IR) were included in the RNA-seq experiment.

#### Quantitative RT-PCR

qRT-PCR using SYBR green assays [18] was performed to validate gene expression levels obtained in RNA-Seq. qRT-PCR on selected genes such as *entS*, *fepC*, enolase, and colicin transporter (colicin-T) was performed using same RNA sample preparation that were used in RNA-Seq, but with different newly generated cDNA. The primers were designed using the Prime-Quest tool (Integrated DNA Technologies, Coralville, IA) (Additional file 5: Table S5) and the cDNA was synthesized from 250 ng of RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). qRT-PCR was performed on CFX touch real-time PCR detection system (Bio-Rad, Hercules, CA) and the *gmk* and *adk* genes of *Salmonella enterica* were used as endogenous control to normalize expression values. Differential gene expression and fold changes were determined using Bio-Rad CFX manager software.

#### Proteome analysis of select protein bands

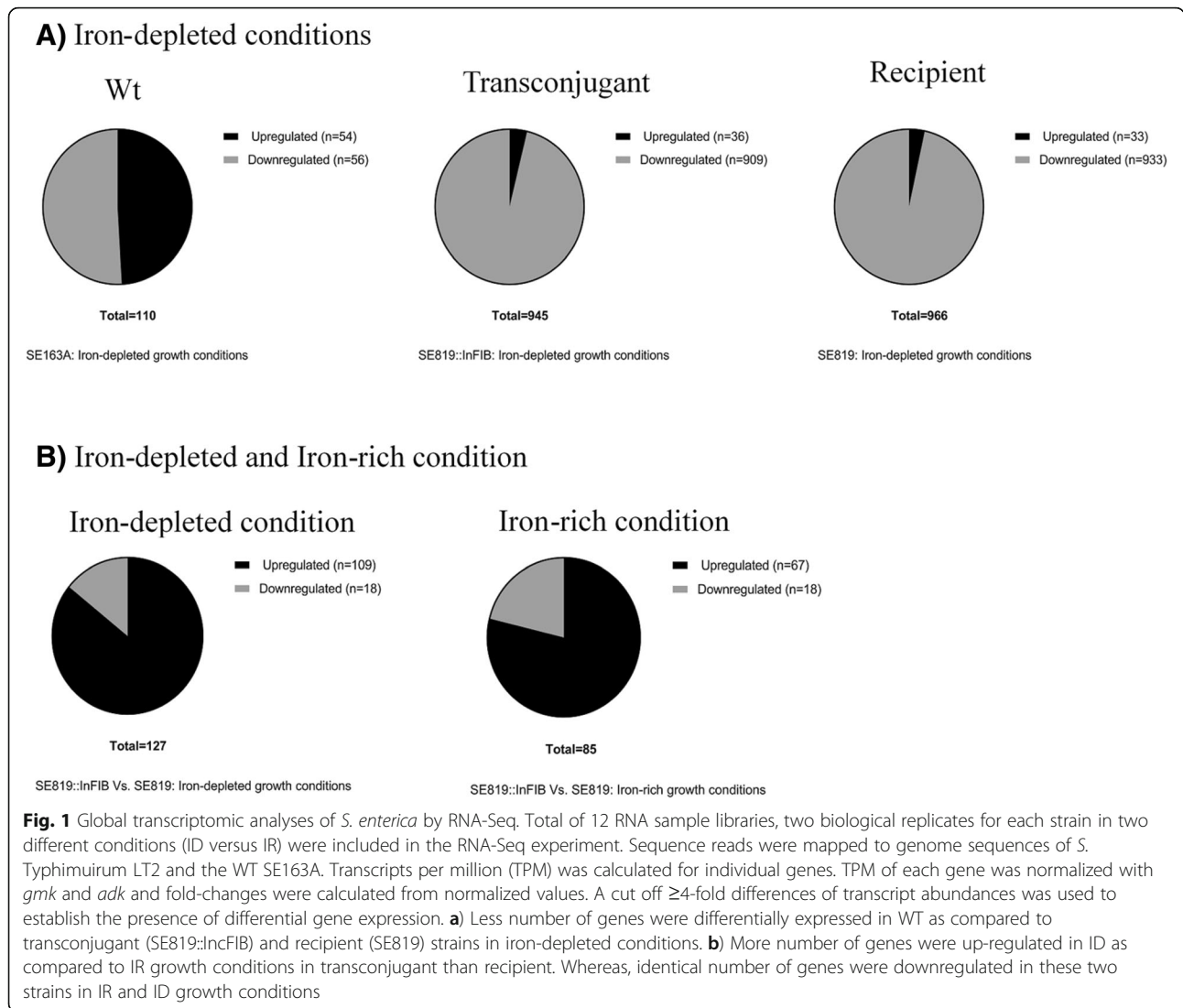
Wild type, recipient, and transconjugant strains were grown in IR and ID LB broth overnight at 37 °C with shaking. Overnight cultures were diluted (1:20) in to corresponding fresh IR and ID LB media and cell lysates were prepared at mid-logarithmic phase. To determine the approximate bacterial cell numbers, optical density (OD) was measured at 600 nm, using a Genesys 10UV spectrophotometer (Thermo Electron Corp., Madison, WI). Bacterial cell pellets were dissolved in protein sample buffer (Bio-Rad, Hercules, CA), boiled at 100 °C for 10 min and 25  $\mu$ L of cell lysates containing equal number of bacterial cells ( $\sim 10^8$  cells) were loaded onto a 4 to 15% gradient Criterion TGX precast protein gel (Bio-Rad, Hercules, CA). Protein bands were separated on a Criterion electrophoresis chamber and visualized by staining with Coomassie blue. Triplicate protein gels were run and stained using protein lysates collected from three independent experiments in order to examine the consistent differential expressions of protein bands from each experiment. Differently expressed protein bands were selected from one representative gel, cut and sent to MS Bioworks (Ann Arbor, MI) for further proteome analyses.

At MS Bioworks, protein content in gel bands were identified by nano-liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described earlier [45].

#### Results

To determine influence of iron on the transcriptomes of WT (SE163A), transconjugant (TC) (SE819::IncFIB) and recipient (SE819) *Salmonella enterica* strains, RNA-Seq analyses were performed using RNA extracted from these strains in IR and ID growth conditions. A goal of the study was to determine the differential gene expression of chromosomally- and plasmid-encoded genes including IncFIB plasmids. Hence, we mapped the sequence reads with the genome sequence of SE163A and *S. Typhimurium* LT2 to accommodate the genes present in WT, TC and recipient strains. The number of differentially expressed genes were substantially higher for recipient ( $n = 966$ ) and TC ( $n = 945$ ) compared to the WT ( $n = 110$ ) strain in ID as compared to IR growth conditions (Fig. 1a). Fifty-four genes were upregulated in ID as compared to IR for the WT strain. These numbers were 36 and 33 for TC and recipient, respectively. Whereas, 56, 909, and 933 genes were downregulated in ID as compared to IR in WT, TC and recipient strains, respectively (Fig. 1a). In ID growth conditions, 109 genes were upregulated whereas 18 genes were downregulated in TC as compared to recipient, while, these numbers were 67 and 18 in IR growth conditions, respectively (Fig. 1b). Gene ID, potential functions and normalized fold changes of differentially expressed genes of WT and TC in ID as compared to IR growth conditions are listed in supplemental document Additional file 1: Table S1, Additional file 2: Table S2, Additional file 3: Table S3 and Additional file 4: Table S4.

Selected genes with higher fold changes that were differentially expressed (upregulated or downregulated) in WT and TC were listed in the Tables 1, 2, 3 and 4. Several virulence associated factors including T3SS, flagellin, cold-shock protein (*cspE*), and other regulatory genes were upregulated in TC in ID compared to IR conditions (Table 1). Whereas, IS1 and *acrR/tetR* transposases, ferritin, sensor protein (*basS/pmrB*), nucleoside triphosphatase (*nudI*), zinc transporter (*zupT*) and several regulatory genes were downregulated in TC in ID conditions (Table 2). For the WT strain, alkylhydroperoxidase (*yciW*), enterobactin transporter (*entS*), iron ABC transporter (*fepCD*), colicin transporter, IncFIB encoded enolase, cyclic di-GMP regulator (*cdgR*) and other regulatory genes were upregulated in ID compared to IR conditions (Table 3). Like TC, IS1 and *acrR/tetR* transposases, ferritin, sensor protein (*basS/pmrB*), zinc transporter (*zupT*), and nucleoside triphosphatase (*nudI*) were also downregulated in ID in WT along with other



genes including ferrous iron transport protein A (*feoA*), and IncFIB encoded toxin gene *artA* (Table 4).

Transcriptomic results obtained from RNA-Seq analyses were validated by qRT-PCR. Transcription of four genes (*entS*, *fepC*, *enolase*, colicin transporter) in WT, transconjugant and recipient (Fig. 2) were examined by qRT-PCR using same RNA preparation that were used for RNA-Seq. All four genes were upregulated with highest fold changes (70-fold) observed for *entS* in WT strain in ID as compared to IR conditions, these data match with RNA-Seq analyses (Fig. 2). These genes were also upregulated in TC and recipient in ID albeit at lower fold-change values as compared to the WT (Fig. 2). Both RNA-Seq and qRT-PCR data demonstrated that the *enolase* gene located on the IncFIB plasmid was upregulated in the WT strain as compared to TC in ID growth conditions.

To examine if iron also influences expression of proteins, proteome analyses of WT, transconjugant and

recipient were performed in IR and ID growth conditions using SDS-PAGE (Fig. 3) and selected differentially expressed proteins in these two conditions were identified by LC-MS/MS analyses (Table 5). Differential protein expressions were observed in these strains in ID as compared to IR as examined by SDS-PAGE (Fig. 3). Our data showed that several proteins were upregulated in ID as compared to IR conditions. Band #1 in SDS-PAGE was upregulated in TC and WT as compared to recipient in ID growth conditions. These protein bands (Band #1), identified as siderophore receptors, IroN and IutA, are located on the chromosome and the IncFIB plasmid, respectively (Table 5). Band #2 in SDS-PAGE was upregulated in TC and recipient as compared to WT (Fig. 3) and was identified as flagellin, FljB (Table 5). Band #3 was only expressed in WT and not in TC and recipient, and was identified as iron acquisition protein, SitA, which is located on the chromosome as well as on the

**Table 1** Selected genes that were upregulated in transconjugant SE819::IncFIB in iron-depleted as compare to iron-rich conditions in RNA-Seq

Gene ID <sup>a</sup>	Gene Function	TCIR – Mean read counts	TCID – Mean read counts	Experiment - Fold Change	Normalized fold change ( <i>gmk</i> & <i>adk</i> )
AY603_00815	alkylhydroperoxidase ( <i>yciW</i> )	2.5	250.0	100.0	48.5
AY603_00965	hydrolase ( <i>ybbA</i> )	96.0	4933.5	51.4	22.4
AY603_14125	inhibitor of glucose transporter ( <i>sgrT</i> )	1.0	42.5	42.5	17.3
AY603_06605	Copper chaperone ( <i>copZ</i> )	9.0	243.0	27.0	12.2
AY603_01300	translation inhibitor protein ( <i>raiA</i> )	140.5	2841.0	20.2	8.7
AY603_23730	ribosome modulation factor	91.5	1849.5	20.2	8.6
AY603_23460	stress-induced protein ( <i>gsiB</i> )	0.5	12.0	24.0	9.0
AY603_22055	type III secretion system protein ( <i>ssaM</i> )	0.5	12.0	24.0	11.4
<i>ssrS</i>	<i>ssrS</i> (6S) RNA interacts with the E-sigma-70	220.0	4016.0	18.3	7.7
AY603_10385	D-alanyl-D-alanine dipeptidase	2.5	39.0	15.6	6.4
AY603_03365	restriction_endonuclease_like	3.5	51.5	14.7	5.9
AY603_05235	cold-shock protein ( <i>cspE</i> )	49.5	642.0	13.0	5.3
AY603_00990	flagellin	108.0	1124.0	10.4	4.2

a

AY603-xxxxx: assigned NCBI annotated ID for WT SE163A

IncFIB plasmid. Lastly, Band # 4 was overexpressed in these three strains in IR as compared to ID conditions. Band #4 was detected as a ribosomal protein L5, RplE.

## Discussion

Iron is an important signaling element that regulates a vast array of genes including virulence factors, as well as an essential growth factor, for many bacterial pathogens to achieve virulence during infection [46–51]. Since bacteria encounter iron limited conditions within the host, bacteria must chelate iron to overcome host sequestration in order to survive and establish infection [21, 51]. Several iron acquisition systems including Sit, aerobactin (Iuc) and Feo system for ferrous iron utilization [34, 52] have been identified in bacteria and located in the chromosome and/or on plasmids. For example, Sit and Iuc have been found to be located on IncFIB plasmids [33, 35]. A previous study demonstrated that deletion of all three iron acquisition systems, namely Sit, Iuc and Feo, abolished the ability of *Shigella flexneri* to form plaque on tissue culture monolayers [53]. The same study showed that a single knock-out of the Sit system resulted in a mutant strain that retained its ability to form plaque similar to WT level while double knockout mutants in different combination such as Sit/IuC or Sit/Feo or Feo/IuC formed smaller plaques as compared to the WT [53]. Other studies have also

shown that deletion of the Sit system, located on the chromosome, resulted in a decreased ability of *S. flexneri* as well as *S. Typhimurium* to cause infection in an animal host [34, 52]. The findings of these studies revealed that all the three iron acquisition systems likely contribute to virulence of *S. flexneri* and related pathogens. We and other investigators demonstrated that IncFIB encoded iron acquisition systems (Sit and aerobactin) likely contribute to increased ability to colonize chickens [54] and increased persistence in human epithelial cells (Caco-2) [18]. Nonetheless, the roles of iron acquisition systems, located on plasmids, in virulence and the mechanisms of how iron regulates iron acquisition genes residing on plasmids of *Salmonella*, has yet to be determined. In the present study, we demonstrated that iron influenced expression of a number of genes located on both chromosome and on the IncFIB plasmid of *S. enterica*, both at the transcriptional and translational levels.

In our previous study we demonstrated that the transconjugant (SE819::IncFIB) persisted in Caco-2 cells at a significantly higher rate than the recipient SE819 after 48 h of post infection when these strains were grown in standard LB or iron-depleted LB, but not in iron-rich LB prior to infection [18]. Additionally, we noticed that WT strain, SE163A, was able to invade and persist in Caco-2 cells at much higher rate than the recipient and TC of *S.*

**Table 2** Selected genes that were downregulated in transconjugant SE819:IncFIB in iron-depleted as compare to iron-rich conditions in RNA-Seq

Gene ID	Gene Function	TCIR-Mean read counts	TCID-Mean read counts	Experiment-Fold Changes	Normalized Fold changes ( <i>gmk</i> & <i>adk</i> )
AY603_25210	transposase ( <i>acrR/tetR</i> )	3907.0	15.5	-252.1	-561.7
AY603_17275	amino acid transporter	1451.5	28.5	-50.9	-123.7
AY603_12100	ferritin	390.5	8.0	-48.8	-112.6
AY603_12215	nitrate/nitrite response regulator ( <i>narP</i> )	685.0	17.5	-39.1	-96.7
AY603_17555	sensor protein ( <i>basS/pmrB</i> )	5266.0	156.5	-33.6	-76.7
AY603_07360	cytoplasmic protein	545.5	19.5	-28.0	-63.7
AY603_25195	ArsR family transcriptional regulator	4757.0	183.5	-25.9	-59.9
AY603_03095	addiction module toxin ( <i>relE</i> )	371.0	16.0	-23.2	-52.0
AY603_20240	nucleoside triphosphatase ( <i>nudI</i> )	2204.0	100.5	-21.9	-50.1
AY603_07740	nitrogen regulatory protein P-II	816.5	40.0	-20.4	-48.9
AY603_25220	IS1 transposase <sup>b</sup>	1807.5	93.5	-19.3	-45.1
AY603_10245	aldehyde-activating protein	3944.0	204.5	-19.3	-44.0
AY603_00540	Nitrous oxide-stimulated promoter	280.5	14.5	-19.3	-43.5
AY603_17515	melibiose:sodium symporter	2567.0	134.0	-19.2	-42.0
AY603_07610	porin	7942.0	488.5	-16.3	-37.2
AY603_21685	sulfur acceptor protein ( <i>csdL</i> )	120.5	8.0	-15.1	-34.2
AY603_24225	MazE_antitoxin	81.5	5.5	-14.8	-34.0
AY603_18780	GTP-binding protein ( <i>ysxC</i> )	321.5	23.5	-13.7	-31.3
AY603_15075	DNA-binding protein ( <i>insE</i> )	698.0	54.0	-12.9	-29.8
AY603_07735	response regulator ( <i>glrR</i> )	350.0	28.0	-12.5	-29.5
AY603_08740	zinc transporter ( <i>zupT</i> )	518.0	45.0	-11.5	-27.8
AY603_06240	phosphonate ABC transporter permease	519.5	42.5	-12.2	-27.5
AY603_24905	repressor-Bacteriophage CII protein	98.0	8.5	-11.5	-25.8
AY603_07050	integrase	2859.5	268.5	-10.6	-24.8
AY603_23620	effector protein ( <i>pipB</i> )	81.0	7.5	-10.8	-25.3
AY603_00645	nitric oxide reductase transcription regulator	293.0	27.0	-10.9	-24.7

*enterica* [18]. To determine how iron levels, influence the gene expression profiles of these three *S. enterica* strains (WT, TC and recipient), we performed transcriptomic analysis in ID and IR growth conditions using RNA-seq. In order to reduce the background gene expression variability among the strains, a cutoff point  $\geq 4$  fold-change differences except for *fepB* and another transporter gene with  $\geq 3$  fold changes (Additional file 3: Table S3), was employed to determine differential gene expression between strains and iron growth conditions. In addition, fold-changes were determined in two different ways; i) experiment fold-changes were determined by exact test using EdgeR by CLC genomic workbench; and ii) fold-changes of gene expression obtained in RNA-Seq were normalized by the gene expression of two housekeeping genes, *gmk* and *adk*. Previous studies confirmed that expression of these genes was stable in different conditions tested, and hence suitable for use as reference genes for normalizations for both RNA-Seq and qRT-PCR analyses [55, 56]. Although we listed both the experiment fold-change and normalized fold-change in Tables 1, 2, 3 and 4; we identified the total number of

genes that were differentially expressed between two groups of strains compared based on the normalized fold changes, as shown in Fig. 1a and b.

RNA-Seq analyses revealed that a higher number of genes were differentially expressed in transconjugant and recipient as compared to the WT strain in ID growth conditions (Fig. 1a and b). This distinct gene expression is likely due to different genetic background of these three-strains studied. WT strain may possess different genetic factors that contribute to increased ability to tightly regulate genes in iron limited conditions in order to deal with iron stress. These characteristics may contribute to increased virulence to WT strain as compared to TC and recipient strains. However, further experimental evidence is needed to substantiate our hypothesis.

More importantly, several genes were differentially expressed in ID and IR conditions in transconjugant as compared to the recipient strain (Fig. 1b). These data indicate that IncFIB encoded factors including iron acquisition systems may influence the gene expression of transconjugant in both ID and IR conditions. Several

**Table 3** Selected genes that were upregulated in WT SE163A in iron-depleted conditions as compare to iron-rich in RNA-Seq

Gene ID	Gene Function	TCIR-Mean read counts	TCID-Mean read counts	Experiment Fold Changes	Normalized fold changes ( <i>gmk</i> & <i>adk</i> )
AY603_00815	alkylhydroperoxidase ( <i>yciW</i> )	2.5	908.5	363.4	336.4
AY603_05415	enterobactin transporter ( <i>entS</i> )	73.5	2918.5	39.7	31.5
<i>rsmC</i>	methyltransferase	169	6176.5	36.5	30.9
AY603_03595	transcriptional regulator ( <i>citB</i> )	19	740	38.9	29.3
AY603_12465	MFS transporter ( <i>uhpC</i> )	73	2169	29.7	23.1
AY603_08910	NAD(P)-dependent oxidoreductase	250.5	6719	26.8	23.3
AY603_14785	leader peptidase ( <i>hopD</i> )	1042	25521.5	24.5	20.5
AY603_05435	O-antigen chain length regulator	78	1833	23.5	20.8
AY603_03600	transcriptional activator ( <i>bglJ</i> )	357.5	7747.5	21.7	17.9
AY603_00970	enterochelin esterase ( <i>fes</i> )	144.5	2860.5	19.8	17.3
AY603_21225	iron ABC transporter permease ( <i>fepD</i> )	30	536	17.9	15.3
AY603_17065	methyl ester esterase	139	1984.5	14.3	12.9
AY603_21220	iron ABC transporter ( <i>fepC</i> )	446	6864	15.4	12.4
AY603_24045	colicin transporter	131.5	1636	12.4	10.7
AY603_22400	cyclic di-GMP regulator ( <i>cdgR</i> )	6	73	12.2	11.3
AY603_24505	enolase <sup>b</sup>	269	2723	10.1	8.9
AY603_24495	camphor resistance protein ( <i>creB</i> ) <sup>b</sup>	1212	11449.5	9.4	8.2
AY603_14430	glucose dehydrogenase	1449.5	11846	8.2	7.6
AY603_03105	anaerobic ribonucleoside triphosphate reductase	305	2584.5	8.5	7.2
<i>zntR</i>	zinc-responsive transcriptional regulator	29.5	194.5	6.6	6.2

virulence associated genes including a T3SS factor (*ssaM*) and cold-shock protein (*cspE*) were upregulated in TC in ID as compared to IR (Table 1). A previous study demonstrated that low pathogenicity strains of *S. Enteritidis* showed distinct transcriptomes with significantly reduced expression of virulence and stress-associated genes compared to the high pathogenicity strains [57].

SPI-2 harbor multiple operons including T3SS-2 genes [58, 59]. T3SS-2 genes facilitate *Salmonella* to survive inside the host cells such as macrophage [60]. In the present study, we observed increased expressions of T3SS-2 genes such as *ssaM* and *sseA* (Table 1 and Additional file 1: Table S1) in ID compared to IR growth conditions in the transconjugant (SE819::IncFIB). Our data supported previous findings reported by Zaharik et al [61] where they demonstrated that SPI-2 genes were upregulated in ID growth conditions as compared IR condition. From these data, we speculate that increased expression of T3SS-2 genes may contribute to increased survival of the transconjugant bacteria in the host cells. However, additional studies are required to substantiate this speculation.

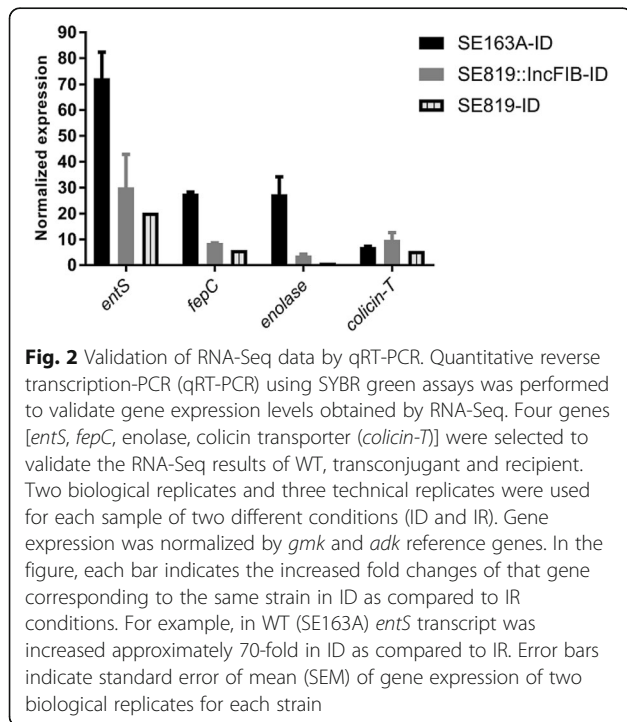
Our data showed that glucose transporter gene *ptsG* (AY603\_16045), a component of phosphotransferase system, was upregulated in WT in ID as compared to IR growth conditions (Additional file 3: Table S3). Several studies demonstrated that *ptsG* is a regulatory gene that control expression of various

virulence associated genes that are often required for the establishment of infection in host [62, 63]. On the other hand, the small regulatory RNA SgrS (AY603\_14125) that is an inhibitor of glucose transporter, (and encodes a small protein SgrT) is upregulated in ID compared to IR in transconjugant (Table 1). A recent study demonstrated that SgrS can perform dual functions, including controlling gene expressions post-transcriptionally via sRNA-mRNA base-pairing interactions and encoding a small protein named SgrT, which can inhibit the transport activity of PtsG [64]. Another small regulatory RNA (sRNA), SsrS (6S RNA) is upregulated in the transconjugant during ID growth conditions (Table 1). This result in agreement with findings in a previous study, where it was shown that 6S RNA in *Burkholderia cenocepacia* upregulated under iron depleted conditions [65]. Another study demonstrated that 6S RNA, encoded by SsrS, plays role in *S. Typhimurium* survival under acid stress [66]. Additionally, the same study demonstrated that a *S. Typhimurium* 6S RNA knockout strain showed reduced invasion ability in HeLa cells and decreased virulence in mouse model [66]. Nonetheless, the mechanisms of how iron limited conditions induce the expressions of sRNAs in WT and transconjugant stains in ID growth conditions in the present study remains to be determined. In the present study, increased expression of the virulence factors in ID conditions in TC but not in recipient may translate



**Table 4** Selected genes that were downregulated in WT SE163A in iron-depleted as compare to iron-rich conditions in RNA-Seq

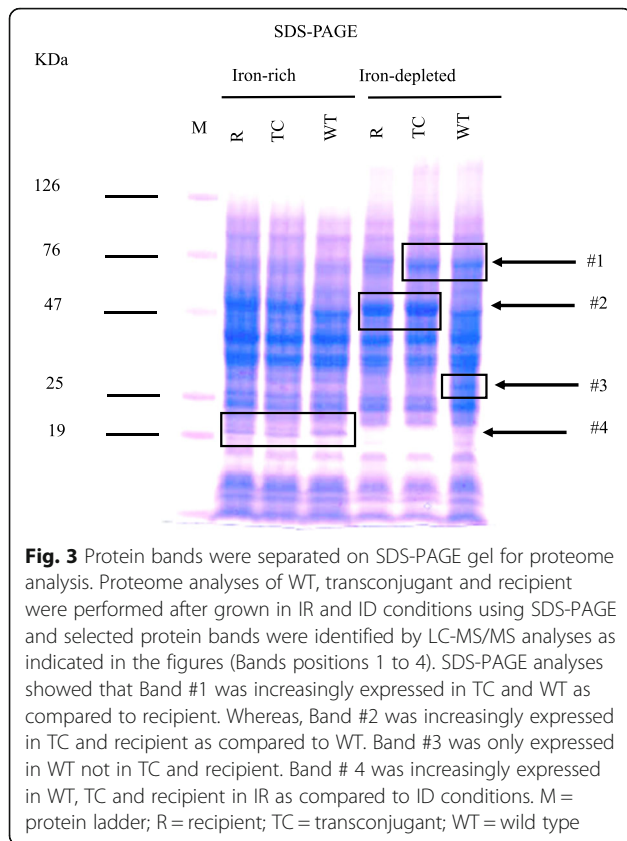
Gene ID	Gene Function	TCIR-Mean read counts	TCID-Mean read counts	Experiment Fold Change	Normalized fold change ( <i>gmk</i> & <i>adk</i> )
AY603_25185	monoxygenase ( <i>hmoA</i> )	14	0.5	-28.0	-13.7
AY603_25210	transposase ( <i>acrR/tetR</i> )	22164	852.5	-26.0	-27.1
AY603_25120	LysR family transcriptional regulator	840.5	40	-21.0	-25.8
AY603_25220	IS1 transposase <sup>b</sup>	14619.5	691.5	-21.1	-23.4
AY603_25115	bicyclomycin/multidrug efflux system-MFS transporter	1130.5	72	-15.7	-24.2
AY603_11055	Mn-containing catalase ( <i>cotJC</i> )	5584.5	561.5	-9.9	-12.4
AY603_22805	DNA-binding protein	489.5	45.5	-10.8	-11.7
AY603_11045	Ferritin-like metal-binding protein ( <i>yciE</i> )	64	9.5	-6.7	-10.4
AY603_16635	glutathione reductase	1374	189.5	-7.3	-7.9
AY603_20240	nucleoside triphosphatase ( <i>nudI</i> )	8717.5	1470	-5.9	-7.2
AY603_11050	Ferritin-like metal-binding protein ( <i>yciE</i> )	97.5	16.5	-5.9	-6.6
AY603_18725	alkaline phosphatases and sulfatases	1016	186	-5.5	-6.7
AY603_07790	nickel transporter ( <i>rcnA</i> )	212.5	37	-5.7	-6.8
AY603_12100	ferritin	932.5	173.5	-5.4	-6.5
AY603_17555	sensor protein ( <i>basS/pmrB</i> )	10343.5	1819	-5.7	-6.5
AY603_15225	toxin ( <i>artA</i> ) <sup>b</sup>	125	24	-5.2	-6.4
<i>feoA</i>	ferrous iron transport protein A	45	8.5	-5.3	-5.5
AY603_03405	Aspartate/glutamate racemase ( <i>racX</i> )	496.5	87	-5.7	-6.1
AY603_08740	zinc transporter ( <i>zupT</i> )	2060	561.5	-3.7	-5.5
AY603_12510	sugar ABC transporter ATP-binding protein	106.5	24	-4.4	-5.8
<i>mltA</i>	murein transglycosylase A	420.5	120	-3.5	-4.8
AY603_02480	aspartate ammonia-lyase	92.5	30	-3.1	-5.7
AY603_09620	acetyltransferase	881	223.5	-3.9	-4.6
AY603_25195	ArsR family transcriptional regulator	14541	4164.5	-3.5	-4.2
AY603_24225	MazE antitoxin/twitching motility protein ( <i>pilT</i> )	105	33.5	-3.8	-4.3



to increased virulence in iron limited conditions in the host during infection; however, the mechanisms of how iron depleted conditions influence regulation of these virulence genes in the TC but not in the recipient, is unknown.

Interestingly, IS1 and *acrR/tetR* transposases encoded on IncFIB plasmid, and chromosome encoded genes including ferritin, *basS/pmrB*, *nudI*, *zupT*, were downregulated in ID conditions both in TC and WT strains (Tables 2 and 4). However, *enolase* (upregulated) and *artA* (down-regulated) genes encoded by the IncFIB plasmid were only differentially expressed in WT but not in TC in ID growth conditions suggesting that IncFIB plasmid encoded genes are likely distinctly regulated in WT (SE163A) as compared to the TC strain background tested ID and IR growth conditions.

A plasmid encoded colicin transporter gene (AY603\_24045) was upregulated ~11 fold in ID compared to IR conditions in WT strain (Table 3). This result is in accordance to a previous finding of Spriewald et al. (2015), where they demonstrated that the ColIb gene (*cib*), was increasingly expressed in iron depleted conditions [67]. A previous study in our laboratory examined the distribution of *cib* in different *Salmonella* serovars isolated from poultry and food producing animals [68]. Our data showed that 35% *Salmonella* isolates



( $n = 32$ ) were positive for *cib* among 92 isolates examined [68]. Ability of colicin production may provide competitive colonization advantage to pathogen including *Salmonella* [69, 70].

Similar to transcriptome analyses, our data showed that the three strains had distinct protein expression profiles as evidenced by proteome analyses of the selected protein bands using SDS-PAGE and LC-MS/MS (Fig. 3). Interestingly, siderophore receptors IroN (product of *iroN* located on the chromosome) and IutA (product of *iutA* located on the IncFIB plasmid) proteins were increasingly expressed in WT and TC in ID conditions as compared to recipient, albeit the recipient lacks the IncFIB plasmid (Fig. 3). Conversely, SitA protein (product of *sitA* gene, located on chromosome as well as on IncFIB plasmid) was expressed only in WT in ID conditions and not in TC and recipient. Sequence analyses showed that the *sitA* located on the chromosome is different than the *sitA* gene located on the IncFIB plasmid [18]. Increased expression of SitA protein located on the IncFIB plasmid in ID conditions may reflect that plasmid-encoded SitA likely plays a role in iron depleted conditions. Nonetheless, the biological functions of plasmid encoded iron acquisition systems, including Sit, are currently unknown. In future studies, we plan to examine the specific functions of plasmid encoded iron acquisition systems in the virulence of *S. enterica*. The proteomic analyses performed on the selective

**Table 5** Selected protein bands of WT, transconjugant and recipient of *Salmonella enterica* were identified by LC-MS/MS

Band position in SDS-PAGE gel <sup>a</sup>	Sample <sup>b</sup>	Approximate Size (KDa)	Identified Protein
#1 <sup>c</sup>	TC-ID	80	IroN (Chromosome) & IutA (IncFIB) (Siderophore receptors)
#1	WT-ID	80	IroN (Chromosome) & IutA (IncFIB) (Siderophore receptors)
#2	R-ID	53	Flagellin (FljB)
#2	TC-ID	53	Flagellin (FljB)
#3 <sup>d</sup>	WT-ID	34	SitA (iron acquisition) (Chromosome and IncFIB plasmid)
#4	R-IR	20	RplE (ribosomal protein L5)
#4	TC-IR	20	RplE (ribosomal protein L5)
#4	WT-IR	20	RplE (ribosomal protein L5)

<sup>a</sup> position of bands was labelled in SDS-PAGE gel (Fig. 3)

<sup>b</sup> WT=wild type; TC=Transconjugant; R=recipient

<sup>c</sup> two most abundant proteins; IroN and IutA were detected.

<sup>d</sup> two different SitA proteins, one is in chromosome and the other is located in the IncFIB plasmid were shown as most abundant proteins in LC-MS/MS analyses

set of protein bands, showed differential expression of proteins (IroN, IutA, FljB, SitA) in WT, transconjugant and recipient were distinct from the expression of these genes at the transcription level observed by RNA-Seq analyses. The reasons for the distinct expression profiles remains unknown and may be explored further in future efforts.

## Conclusion

Overall, the present study provides important findings as to how iron influences different genes of *S. enterica* at the transcriptional and translational levels in isolates that carry iron acquisition systems encoded both on the chromosome and on plasmids such as IncFIB in this study (WT and TC). Based on the findings of the present study, we hypothesize that plasmid encoded iron acquisition systems may be associated with biological functions distinct to that of chromosome encoded iron acquisition systems. Nonetheless, further study is necessary to identify the specific functions of plasmid-encoded factors including iron acquisition systems in the virulence of *Salmonella*. The present study further supports the necessity of understanding the roles of plasmid-encoded factors in virulence and pathogenesis of *Salmonella*.

## Additional files

**Additional file 1: Table S1.** List of genes that were upregulated in TC in ID as compared to IR growth conditions. (XLSX 4483 kb)

**Additional file 2: Table S2.** List of genes that were down regulated in TC in ID as compared to IR growth conditions. (XLSX 4349 kb)

**Additional file 3: Table S3.** List of genes that were upregulated in Wt in ID as compared to IR growth conditions. (XLSX 39 kb)

**Additional file 4: Table S4.** List of genes that were down regulated in Wt in ID as compared to IR growth conditions. (XLSX 39 kb)

**Additional file 5: Table S5.** Primers used in qRT-PCR for validation of RNA-Seq data. (DOCX 12 kb)

## Abbreviations

Coli-T: Colicin transporter gene; ID: Iron-deplete; IncFIB: Incompatibility group (Inc) FIB plasmid; IR: Iron-rich; qRT-PCR: Quantitative reverse transcription polymerase chain reaction; R: Recipient; *S. enterica*: *Salmonella enterica*; TC: Transconjugant; Wt: Wild type

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## Availability of data and materials

The whole genome sequences of *S. enterica* WT SE163A and the recipient SE189 were publicly available at NCBI under the accession numbers LSZD00000000 and LSZE00000000, respectively.

## Author's contributions

BK, SF: designed and coordinated the study, carried out experiments and wrote the manuscript; JX, CG, AO, PR: contribute in RNA-Seq analysis and edited the manuscript; All authors read and approved the submitted manuscript.

## Ethics approval and consent to participate

Not Applicable.

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