Genotypic and phenotypic characterization of multidrug resistant *Salmonella* Typhimurium and *Salmonella* Kentucky strains recovered from chicken carcasses

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Abstract

*Salmonella* Typhimurium is the leading cause of human non-typhoidal gastroenteritis in the US. *S. Kentucky* is one the most commonly recovered serovars from commercially processed poultry carcasses. This study compared the genotypic and phenotypic properties of two *Salmonella enterica* strains Typhimurium (ST221_31B) and Kentucky (SK222_32B) recovered from commercially processed chicken carcasses using whole genome sequencing, phenotype characterizations and an intracellular killing assay. Illumina MiSeq platform was used for sequencing of two *Salmonella* genomes. Phylogenetic analysis employing homologous alignment of a 1,185 non-duplicated protein-coding gene in the *Salmonella* core genome demonstrated fully resolved bifurcating patterns with varying levels of diversity that separated ST221_31B and SK222_32B genomes into distinct monophyletic serovar clades. Single nucleotide polymorphism (SNP) analysis identified 2,432 (ST19) SNPs within 13 Typhimurium genomes including ST221_31B representing Sequence Type ST19 and 650 (ST152) SNPs were detected within 13 Kentucky genomes including SK222_32B representing Sequence Type ST152. In addition to serovar-specific conserved coding sequences, the genomes of ST221_31B and SK222_32B harbor several genomic regions with significant genetic differences. These included phage and phage-like elements, carbon utilization or transport operons, fimbriae operons, putative membrane associated protein-encoding genes, antibiotic resistance genes, siderophore operons, and numerous hypothetical protein-encoding genes. Phenotype microarray results demonstrated that ST221_31B is capable of utilizing certain carbon compounds more efficiently as compared to SK222_32B; namely, 1,2-propanediol, M-inositol, L-threonine, α-D-lactose, D-tagatose, adonitol, formic acid, acetoacetic acid, and L-tartaric acid. ST221_31B survived for 48 h in macrophages,
accession numbers of SRS775470 and SRS775521, respectively.

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while SK222_32B was mostly eliminated. Further, a 3-fold growth of ST221_31B was observed at 24 hours post-infection in chicken granulosa cells while SK222_32B was unable to replicate in these cells. These results suggest that *Salmonella* Typhimurium can survive host defenses better and could be more invasive than *Salmonella* Kentucky and provide some insights into the genomic determinants responsible for these differences.

### Introduction

Salmonellosis, caused by the Gram-negative bacterial genera, *Salmonella*, is one of the major causes of bacterial food-borne illness globally. Each year, an estimated 93.8 million cases of salmonellosis occurs worldwide [1]. In the United States, salmonellosis is the second leading cause of food-borne illnesses in humans [2], accounting for approximately 1 million cases, 19,336 hospitalizations and 378 deaths each year [2]. About 31% of all food-borne illness related deaths each year in the US are attributed to *Salmonella* infections [3] and the annual medical cost for salmonellosis in the US is estimated at $3.7 billion [4].

*Salmonella enterica*, belonging to the family, Enterobacteriaceae, consists of more than 2,500 serovars. *Salmonella* Enteritidis and S. Typhimurium are the two most common serovars that cause food-borne illnesses in the US [2]. However, S. Kentucky is one of the most frequently isolated serovars from poultry carcasses (ST152) in the US [3] and is sometimes attributed with one or more antibiotic resistant phenotypes [6–8]. Historically, S. Kentucky has rarely been associated with human illness; however, recently S. Kentucky (ST198) has been found to be frequently associated with human clinical cases in travelers to North Africa and South Asia [9–10]. Moreover, the global emergence of multidrug resistant *Salmonella* strains poses a serious threat to public health and has drawn significant interest to poultry industries, particularly in the poultry processing steps to control the level of contamination and transmission [11].

Among the various strategies to control the growth of *Salmonella* and other pathogenic microorganisms, chlorine in the form of sodium hypochlorite (HOCl), is used as a safe antimicrobial agent in commercial poultry processing plants during the immersion-chilling step. Sodium hypochlorite at a lower concentration (20 to 50 ppm) can kill *Salmonella* and other pathogens [12]. In addition, commercial poultry industries also used to treat these birds with various broad-spectrum antibiotics to prevent subclinical infections and to reduce the overall bacterial load in these birds [13]. Due to the growing concern of emerging resistant organisms, final guidelines were issued by the U.S. Food and Drug Administration (2013) to phase out the use of medically important antibiotics in livestock for production purposes [14]. Although antibiotic use is being phased out, current practice still allows for the use of broad-spectrum antibiotics in broiler flocks.

Recent reports on the isolation of multidrug resistant (MDR) S. Typhimurium and S. Kentucky isolates from commercially processed chicken carcasses raises concerns regarding the management practices used in this industry [6, 15–16]. How these *Salmonella* isolates survive chilling and chlorine treatment is an unanswered question. Of further concern is that some of these recovered *Salmonella* isolates, regardless of which serovar they belonged to, displayed resistance to ceftriaxone (51.7%), a commonly used chemotherapeutic for *Salmonella* infected patients [6, 17]. Taken together, one has to question whether the use of antibiotics in the poultry industry is being managed effectively and the efficacy of chlorine treatment in controlling multidrug resistant *Salmonella* during processing.
In a previous study [6], our laboratory conducted research on the prevalence and antimicrobial resistance of *Salmonella* spp. isolated from processed poultry. Whole broiler carcasses were obtained directly from the downstream processing line at two selected points (pre- and post-chill) from a commercial processing plant. A significant number of recovered *Salmonella* isolates (45.8%) were resistant to an average of five or more antibiotics [6], and these isolates were further analyzed by PCR assays for selected virulence markers [16]. However, information is limited on the genotypic, phenotypic and immunologic properties of MDR *S. Typhimurium* and *S. Kentucky* strains surviving the immersion-chilling step in a commercial poultry processing plant. Therefore, this study was undertaken to identify similarities and differences between representative MDR *S. Typhimurium* and MDR *S. Kentucky* strains, using whole genome sequencing and comparative genomic analyses, phenotypic microarray, and macrophage killing and granulosa cell invasion. Characterization and comparison of these traits should lead to a better understanding of the processes that drive the evolution of this species through the loss of genes or acquisition of mobile genetic elements.

**Materials and methods**

**Selection of strains and extraction of DNA for whole genome sequencing**

*Salmonella* strains used in this study were isolated in a previous study [6] from commercially processed whole broiler carcasses collected from a processing plant in the mid-Atlantic region. A total of 309 *S. Typhimurium* (*n* = 75) and *S. Kentucky* (*n* = 234) isolates were recovered by Parveen et al. [6] and 89.5% of *S. Typhimurium* and 84.2% of *S. Kentucky* isolates were multidrug resistant. Among these characterized *Salmonella* strains [6], one multidrug resistant (MDR), post-chill *S. Typhimurium* (ST221_31B) and one MDR, post-chill *S. Kentucky* (SK222_32B) strains were selected for this study as a representative strain from these two serovars, as most of the isolates were MDR and all isolates contained virulence genes *invA* and *pagC*. Both strains used in this study, recovered from the same chicken carcass, were resistant to six different antibiotics: tetracycline, ampicillin, amoxicillin, cefoxitin, ceftiofur, and sulfisoxazole [6, 16].

**DNA extraction**

*Salmonella enterica* strains ST221_31B and SK222_32B were sub-cultured from frozen stocks onto Tryptic Soy Agar (TSA) agar plates amended with 5% sheep blood and incubated overnight at 37°C. Single isolated colonies were inoculated into Tryptic Soy Broth (TSB) and incubated overnight at 37°C, with shaking. Cell pellets were harvested by centrifugation at 6000 x g for 5 min, and genomic DNA was extracted with the QIAcube automated sample preparation platform, using the QIAamp DNA mini protocol (Qiagen, Valencia, CA, USA).

**Sequencing of Salmonella genomes**

Extracted genomic DNA was quantified using a Qubit 2.0 Flurometer (Life Technologies (ThermoFisher, Waltham, MA, USA), diluted, and prepared for sequencing using the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA). Whole genome sequencing was performed on the MiSeq platform (Illumina, San Diego, CA, USA), utilizing 500 cycles of paired-end reads. Fastq datasets were trimmed and de novo assembled with CLC Genomics Workbench version 7.0 (CLC bio, Aarhus, Denmark). The draft genome size of *S. Typhimurium* ST221_31B is 4,989,491 bp, on 72 contigs, with a G+C% content of 52.1, while the draft genome size of *S. Kentucky* SK222_32B is 5,005,607 bp, on 55 contigs, with a G+C% content of 51.9. The genome coverage at each position for *S. Typhimurium* ST221_31B and *S.
Kentucky SK222_32B was, on average, 69 and 75%, respectively. The draft genome sequence assemblies were annotated using Rapid Annotation using Subsystem Technology (RAST) [18].

Data availability and accession numbers
The Whole Genome Shotgun projects for S. Typhimurium ST221_31B and S. Kentucky SK222_32B have been deposited at DDBJ/ENA/GenBank under the accession JUIT01000000 and JIUU01000000, respectively. The versions described in this paper are versions JUIT0100000.1 and JIUU0100000.1. The genome sequencing reads for S. Typhimurium ST221_31B and S. Kentucky SK222_32B have been deposited at the NCBI SRA under the accession numbers of SRS775470 and SRS775521, respectively.

Phylogenomics
A phylogenetic tree of the two newly sequenced Salmonella strains was constructed with 28 previously sequenced S. enterica strains to establish evolutionary relationships. Orthologous regions were identified by comparison based on similarity and the set of orthologous regions for each Open Reading Frame (ORF) of a reference genome was identified according to nucleotide similarity, and aligned using the CLUSTALW2 program. The resultant multiple alignments were concatenated to form genome scale alignments, and resultant alignments were used to construct the neighbor-joining tree [19]. Maximum likelihood [20] and maximum parsimony [21] trees were also constructed. Single nucleotide polymorphisms (SNPs) analysis of S. Typhimurium strains and S. Kentucky strains was determined using Parsnp core genome multi-aligner from the Harvest SNP calling package [22].

Comparative genome analysis
Two methods for whole genome comparison were used to identify genomic similarities and differences between newly sequenced strains with previously sequenced S. enterica reference genomes including plasmids [23]. A sequence based genome-to-genome comparison was carried out using the bidirectional BLASTP strategy of the RAST SEED Viewer [24], version 2.0, to compare strains ST221_31B and SK222_32B with S. arizonae (IIIa) 62:z4,z23:: strain RKS2980 (GenBank Accession NC_010067.1), S. Newport SL254 (GenBank Accessions NC_011080.1, NC_011079.1, and NC_009140.1), S. Paratyphi B SPB7 (GenBank Accession NC_010102.1), S. Agona SL483 (GenBank Accessions NC_011149.1 and NC_011148.1), S. Typhi CT18 (GenBank Accessions NC_003198.1, NC_003384.1 and NC_003385.1), S. Choleraesuis SC-B67 (GenBank Accessions NC_006905.1, NC_006856.1 and NC_006855.1), S. Dublin CT_02021853 (GenBank Accessions NC_011205.1 and NC_011204.1), S. Enteritidis P125109 (GenBank Accession NC_011294.1), S. Gallinarum 287/91 (GenBank Accession NC_011274.1), S. Heidelberg SL476 (GenBank Accessions NC_011083.1, NC_011082.1 and NC_011081.1), S. Kentucky CVM29188 (GenBank Accessions ABAK0200001.1, CP001122.1, CP001121.1 and CP001123.1), and S. Typhimurium LT2 (GenBank Accessions NC_003197.1 and NC_003277.1). A subset of this comparison is shown in “S2 and S3 Figs”. Due to the graphical output limitations of the SEED Viewer software, only 9 (RKS2980, SL254, SC-B67, CT_02021853, P125109, 287/91, SL476, CVM29188 and LT2) out of 12 genomes were used for the comparison shown in “S2 and S3 Figs”. Additionally, the comparative distribution of Salmonella virulence determinants was carried out by querying the genomes against a web-based virulence factor database (VFDB) (http://www.mgc.ac.cn/VFs/), and the genome assemblies were submitted to the PlasmidFinder tool from CGE to elucidate plasmid incompatibility classes [25].
Phenotype characterizations by Biolog phenotype microarray (PM)

Phenotypic microarrays (PM) of both strains were prepared according to the manufacturer’s specifications (Biolog, Inc., Hayward, CA). *Salmonella* Typhimurium (ST221_31B) and *S*. Kentucky (SK222_32B) were incubated overnight at 37°C on tryptic soy agar (TSA) plates. Sterile swabs were used to aseptically transfer cells from the TSA plates to tubes containing 16 ml of inoculation fluid-0 (IF-0) until a turbidity transmittance (T) of 42% ± 1 was reached on the Biolog turbidity meter. A 1:5 dilution of the 42% T into IF-0+dye was performed to give a final cell density of 85% T. PM assays consisted of 10 panels (PM1, PM2A, PM3B, PM4A, PM5, PM6, PM7, PM8, PM9, PM10) which tested the strains’ ability to utilize different carbon, nitrogen, phosphorus, sulfur, and peptide nitrogen sources, as well as varying osmolytes, nutrient supplements and pH conditions. Antibiotic susceptibility assays for PM were not done, since both strains were tested for their sensitivity to 15 different antimicrobials using traditional antimicrobial sensitivity testing [6]. After the cell suspensions were added to each PM plate, they were incubated for 48 hours in the OmniLog reader (Biolog). Bacterial growth, measured by the reduction of tetrazolium dye, was recorded every 15 minutes by the OmniLog charge coupled device analyzing camera. PM data analysis was performed using OmniLog® Phenotype™ MicroArray Software Release 1.2.

Infection of cultured macrophages and assessment of intracellular growth of bacteria

*Salmonella* Typhimurium (ST221_31B) and *S*. Kentucky (SK222_32B) were grown overnight at 37°C in BHI (Brian heart infusion) broth in static culture and then washed twice with PBS (phosphate buffered saline, pH 7.4). The optical density (at 600 nm) was measured to adjust the bacterial cell density to 10^8 colony forming units/ml (CFU/ml) and the bacterial suspension was further diluted in DMEM (Dulbecco’s Modified Eagle Medium). The bacterial suspension was added to 12-well plates at a multiplicity of infection (MOI) of 10 for both HD-11 and RAW 264.7 cells (ATCC TIB-71, Manassas, VA). HD-11 cells are a macrophage-like immortalized cell line derived from chicken bone marrow and transformed with the avian myelocytomatosis type MC29 virus [26]. Plates were centrifuged for 5 min to synchronize the infection followed by incubation at 37°C under 5% CO_2 for 90 min for both macrophage cell lines. Subsequently, plates were washed three times with pre-warmed PBS (pH 7.4) and were incubated with media containing 100 μg/ml gentamicin for 1 h, followed by culture with DMEM containing 50 μg/ml gentamicin for the rest of the experiment. Intracellular killing of bacteria by macrophages was assessed by means of the standard gentamicin assay [27–28]. At the end of the infection period, the infected macrophages were treated with 100 μg/ml gentamicin to kill the extracellular bacteria. At 0 h, 24 h and 48 h of incubation after the start of infection, the supernatant was removed and cells were washed three times with DMEM and lysed with 0.1% Triton X-100. Serial dilutions of the lysates were plated onto BHI agar to allow colony formation for quantification of intracellular bacteria.

Invasion of chicken ovarian granulosa cells and assessment of intracellular growth of bacteria

The assay procedure used for invasion of granulosa cells by *Salmonella* strains was as previously described [29] with minor modifications. Although ST221_31B and SK222_32B are isolates from broiler carcasses, *S*. Typhimurium is known to colonize poultry and contaminate eggs, and as such there are no egg related outbreaks associated with *S*. Kentucky. Briefly, actively laying hens were euthanized by cervical dislocation, and pre-ovulatory follicles were excised from the ovarian tissue. All animal procedures described herein were reviewed and
approved by the Center for Food Safety and Applied Nutrition (CFSAN) committee for Institutional Animal Care and Use (IACUC). The granulosa layer was separated from the thecal layer as previously described [30] and dissociated in Type IV collagenase (1.5 mg/ml, Worthington Biochemical Corp., Lakewood, NJ, USA) containing M199 medium (Life Technologies, Grand Island, NY, USA). The viable dispersed cells were enumerated by trypan blue exclusion method and suspended in M199 medium supplemented with Bovine Serum Albumin (0.2%), Glucose (0.2%), Chicken Serum (4%), Fetal Bovine serum (4%) and Antibiotic-Antimycotic (1X) solutions (Life Technologies). Approximately 2×10^6 granulosa cells were seeded onto 60 mm cell culture dishes (Corning, Tewksbury, MA, USA), and grown to form a monolayer for 48 h at 37˚C with 5% CO_2. Logarithmic cultures of *Salmonella* strains (ST221_31B and SK222_32B) grown in aerated buffered peptone water at 37˚C were normalized at OD 600 nm to 2×10^8 CFU/ml. The ovarian granulosa cells (2×10^6) in antibiotic-free medium were infected with *Salmonella* strains at a MOI of 30. The cells were incubated for 1.5 h at 37˚C and rinsed with gentamicin containing medium (50 μg/ml, Life Technologies). Non-internalized bacteria were removed by washing and killed by gentamicin treatment. After 1 h, cells were incubated at a lower gentamicin concentration (25 μg/ml) for a total of either 4 h or 24 h post infection. After infection, granulosa cells were again enumerated, washed with gentamicin-free medium and lysed with 0.2% Triton X-100. The intracellular bacteria were collected and enumerated on Xylose Lysine Desoxycholate agar using an Eddy Jet 2 spiral plater and flash & go colony counter (Neutec Group, Inc. Farmingdale, NY, USA). *Salmonella* counts were expressed as colony forming units per granulosa cells lysed (CFU/granulosa cell).

Colony forming unit count data are from one representative experiment done at least 4 times. Percent survival data were pooled from 3–4 experiments. Data were mean ± SEM from representative experiment (p < 0.05 by t-test, unpaired).

**Results**

**Phylogenomics of S. Typhimurium (ST221_31B) and S. Kentucky (SK222_32B)**

The genomes of the two newly sequenced strains ST221_31B (ST19 [Sequence Type 19], EBG1 [eBURST group 1]) and SK222_32B (ST152, EBG15) were compared with 28 previously sequenced, publicly available *Salmonella* genomes ("Table 1") and a phylogenetic tree was constructed. The phylogeny of all 30 *S. enterica* strains were inferred by constructing a genetic relatedness neighbor-joining tree using homologous alignment of orthologous 1,185 highly conserved protein coding genes, representing approximately 1.1 million base-pairs (~1,113,790 bp) of a *Salmonella* core genome. The tree was rooted with *Salmonella enterica* subsp. arizonae serovar 62:z4,z23:-str. RSK2980 ("Fig 1"). The rooted *Salmonella* tree demonstrated fully resolved bifurcating patterns with varying levels of diversity and placed both *S. enterica* serovars Typhimurium and Kentucky strains into distinct monophyletic clades, suggesting that these two *S. enterica* serovars evolved into distinct evolutionary lineages from their most common phylogenetic ancestor. The newly sequenced Typhimurium strain (ST221_31B) branched with other Typhimurium genomes, LT2 and CVM23701, with *S. enterica* serovar Saintpaul (SARA23) being the closest phylogenetic out-group. This observation indicated that serovar Typhimurium shared a common ancestry with serovar Saintpaul before these two serovars evolved independently. Similarly, the newly sequenced Kentucky strain (SK222_32B) formed a tight monophyletic clade with two other Kentucky genomes, CDC191 and CVM29188 ("Fig 1"). *S. enterica* serovars Agona (SL483) and Weltevreden (HL_N05-537) appeared as out-groups indicating their shared ancestry with serovar Kentucky. Maximum likelihood and maximum parsimony trees are shown in "S1(A) and S1(B) Fig" respectively.
To elucidate genomic differences between genomes within each serovar cluster, entire genomes were compared for high resolution SNPs analysis. 2,432 (ST19) SNPs were identified among the *S. Typhimurium* ST221_31B, 14028 S, LT2, T000240, ST1660/06, DT104, YU15, SARA13, SL1344, ST4/74, UK-1, CVM23701 and 08–1736 genomes (“Fig 2A”), whereas 650 (ST152) SNPs were found among the *S. Kentucky* SK222_32B, 22694, CVMN51313, CVM29188, CDC191, ARS-CC515, ARS-CC6181, 5349, CFSAN011775, 13562, CVMN50435, ABBSB1008-2 and SA20030505 genomes (“Fig 2B”). In general, SNPs were distributed stochastically within Kentucky and Typhimurium genomes, without indication of mutational hotspots (data not shown).

### Comparative genomics of ST221_31B and SK222_32B

General features of the newly sequenced ST221_31B and SK222_32B genomes are given in “S1 Table”. Bi-directional BLAST approach was used to compare ST221_31B and SK222_32B.

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**Table 1. List of strains used for phylogenetic analysis of *S. enterica* genomes.**

| Strain      | Serovar                | Source Type                                      | Accession no.     | Sequence type |
|-------------|------------------------|--------------------------------------------------|------------------|---------------|
| ST221_31B*  | Typhimurium            | Processed whole chicken carcase                   | JUIT01000000     | ST-19         |
| LT2         | Typhimurium            | Human                                            | AE006488         | ST-19         |
| CVM23701    |                        | I4, _5_,12:i:__                                  | ABAO00000000     | ST-19         |
| SK222_32B*  | Kentucky               | Processed whole chicken carcase                   | JUII01000000     | ST-152        |
| CDC 191     | Kentucky               | Human                                            | ABEI00000000     | ST-152        |
| CVM29188    | Kentucky               | Chicken breast                                   | ABAK00000000     | ST-152        |
| SL483       | Agona                  | Human                                            | CP001138         | ST-13         |
| SL477       | Dublin                 | Human                                            | CP001144         | ST-10         |
| RI_05P066   | Hadar                  | Human                                            | ABFG00000000     | ST-33         |
| SL476       | Heidelberg             | Ground turkey                                    | CP001120         | ST-15         |
| SL486       | Heidelberg             | Human                                            | ABEL00000000     | ST-15         |
| SL474       | I4, _5_,12:i:__        | Human                                            | ABAO00000000     | ST-19         |
| SL478       | Javiana                | Human                                            | ABEH00000000     | ST-24         |
| SL254       | Newport                | Human                                            | CP001113         | ST-45         |
| SL317       | Newport                | Human                                            | ABEW00000000     | ST-5          |
| SARA23      | Saintpaul              | Human                                            | ABAM00000000     | ST-50         |
| SARA29      | Saintpaul              | Human                                            | ABAN00000000     | ST-95         |
| CVM19633    | Schwarzengrund         | Dehydrated chili                                 | CP001127         | ST-322        |
| SL480       | Schwarzengrund         | Human                                            | ABEJ00000000     | ST-322        |
| SL491       | Virchow                | Human                                            | ABFH00000000     | ST-16         |
| HI_N05-537  | Weltevreden            | Scallops                                         | ABFF00000000     | ST-365        |
| RKS2980     | 62:z4,2z:23:          | NA                                               | CP000880         | NA            |
| SC-B67      | Choleraesuis           | Human                                            | AE017220         | ST-66         |
| P125109     | Enteritidis            | Human                                            | AM933172         | ST-11         |
| 287/91      | Gallinarum             | Chicken                                          | AM933173         | ST-331        |
| AKU_12601   | Paratyphi A            | Human                                            | CP000026         | ST-85         |
| ATCC 9150   | Paratyphi A            | Laboratory strain                                | CP000026         | ST-85         |
| SPB7        | Paratyphi B            | Human                                            | CP000886         | ST-307        |
| RKS4594     | Paratyphi C            | Human                                            | CP000857         | ST-114        |
| CT18        | Typhi                  | Human                                            | AE014613         | ST-2          |
| Ty2         | Typhi                  | NA                                               | AE014613         | ST-1          |

*Strains sequenced in this study; NA, not available.

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genomes with nine previously sequenced Salmonella genomes ("S2 and S3 Figs", respectively). In each figure ("S2 and S3 Figs"), colored arrows indicate examples of dissimilar gene content, such as insertion of mobile genetic elements (e.g., transposons, integrons) or plasmids in the reference genome, either ST221_31B, or SK222_32B, respectively, or deletion of genomic islands in one to all of the comparison genomes. In addition, a number of dissimilar smaller
genomic islands, such as smaller prophage and prophage-like elements, characterized operons, and hypothetical proteins were also identified in both ST221_31B and SK222_32B strains. Detailed information is given in “S2 and S3 Tables”, respectively.

When the genomes of ST221_31B and SK222_32B were compared with a panel of twelve diverse Salmonella genomes, we identified 66 genomic regions that were present in both new genomes but missing from at least one comparison genome. Not surprisingly, the majority (59) of these dispensable genetic elements were found to be missing in the genome of Salmonella enterica subsp. arizonae strain RKS2980. These included the genetic determinants of phenotypic traits used to distinguish subsp. enterica, from this subspecies; namely, L(+)-tartrate and galactitol utilization, as well as operons for the utilization of several other putative carbon substrates, including allantoin, D-galacturonate and D-glucuronate, glycerate, L-ascorbate, L-idionate, glucosamine, mannose, gentisate, L-rhamnionate, and xanthosine, stfABCDEFG and stdABCD π-fimbriae, safABCD γ3-fimbriae, lpfABCDE and sthABCDE γ1-fimbriae, and β-fimbriae clusters, and several membrane-associated and stress-response proteins. The genome of Salmonella enterica subsp. enterica Typhi CT18 was also missing several [29] of the genomic regions identified.

A total of 20 genomic regions were identified in the genome of ST221_31B, but absent in SK222_32B. These included three prophages, three prophage-like elements and two large integrons. We assume that most of the hypothetical or poorly annotated proteins encoded in these elements are phage-related proteins and toxin/antitoxin pairs; however, one of the phage-like elements encodes the O-antigen acetylase gene, oafA, responsible for the O5 epitope. For ST221_31B, there is a frameshift mutation in this gene, which results in loss of function, indicative of the Copenhagen variant of Typhimurium [31]. In addition, operons responsible for the utilization of D-tagatose, D-glucitol/D-sorbitol and L-sorbose, D-galactonate, and myo-inositol, and a fructose-like phosphotransferase system (PTS) were present in the genome of ST221_31B, as well as the cluster of genes that confer the ability to utilize sialic acid. Twenty
two genomic regions were identified in the genome of SK222_32B, which were absent from the genome of ST221_31B. Among these, we identified one prophage, and five prophage-like elements. One of these elements harbors a four gene operon that is responsible for the DNA degradation phenotype, dndBCDE. The presence of this functional cluster results in strains that are non-typeable by pulsed-field gel electrophoresis (PFGE) due to degradation of the DNA. We also identified a transposon, which harbors a κ-fimbriae gene cluster (pef). In addition to these mobile genetic elements, we identified two additional fimbriae clusters, the steABCDEF π-fimbriae and the staABCDEF γ4-fimbriae, and gene clusters encoding a "cryptic" mannitol transport system, the ydf unknown carbohydrate utilization system, and an arsenic resistance pump.

A total of five plasmids were found; two were found in the genome of ST221_31B and three in the genome of SK222_32B. A homologous IncI incompatibility class plasmid of approximately 100 kbp in size was found in the genomes of ST221_31B and SK222_32B. This plasmid was highly similar (99% identity, 99–100% query coverage) to plasmids from other Salmonella isolates collected from poultry; namely pN13-1290_98 (serovar Heidelberg, turkey meat, GenBank Accession CP012936.1), pCVM29188_101 (serovar Kentucky, poultry, GenBank Accession CP001121.1), pSA02DT1068701_99 (serovar Heidelberg, turkey meat, GenBank Accession CP012923.1), p12-4374_96 (serovar Heidelberg, turkey meat, GenBank Accession CP012929.1), with each plasmid displaying slight differences, apparently driven by transposases and insertion sequences ("S4B Fig"). Interestingly, this plasmid group is also highly similar to plasmid pSTM709 (GenBank Accession NC_023915), which harbors beta lactamase CMY-2, from a serovar Typhimurium isolate that is believed to be the first Amp-C producing S. Typhimurium strain in Uruguay. Phylogenetic reconstruction of the nucleotide sequence of this plasmid ("S4A Fig") indicates a high degree of mobility, as its phylogeny is completely inconsistent with the Salmonella core genome phylogeny ("Fig 1"). This shared conjugative plasmid harbors the bla CMY-2 gene, conferring resistance to cephalosporins, the blc gene, and the sugE gene, which confers resistance to quaternary ammonium compounds, in a gene cassette flanked by mobility genes [32].

In addition to the shared IncI plasmid described above, strain ST221_31B harbors an IncA/C2 incompatibility class plasmid, which is estimated at 106,610 bp in size. It is contained on two contigs, ctgs. 2 and 56, which have a 20 bp overlap. It was found to be most similar, in terms of nucleotide identity, to an unnamed plasmid from the genome of S. Typhimurium strain CFSAN001921 (GenBank Accession CP006050.1), which was also isolated from chicken breast [33]. However, the plasmid from strain ST221_31B is an estimated 116,358 bp smaller in size than that of strain CFSAN001921, missing this segment as one large piece of DNA located on the 3’ end. Present on this plasmid are a tetRA gene cluster and a mercury resistance operon.

In addition to the shared IncI plasmid described above, strain SK222_32B harbors an IncFIB/IncFIIA (two origins of replication) incompatibility class plasmid, which is nearly identical to plasmid CVM29188_146 (GenBank Accession CP001122.1), and an IncX1 incompatibility class plasmid, which is nearly identical to plasmid pCVM29188_46 (GenBank Accession CP001123.1) [32]. Of interest, genes responsible for streptomycin and tetracycline resistance are encoded in the larger F-type conjugative plasmid, along with two siderophore gene clusters, aerobactin and salmonchelin, and the sitABCD-encoded manganese ACB transporter.

Virulence determinants found in both ST221_31B and SK222_32B are shown in “Table 2” (Sequence and locus tag of these virulence factors can be accessed at http://www.mgc.ac.cn/VFs).
Table 2. Virulence determinants of ST221_31B and SK222_32B, as determined by querying each genome against the virulence factors of pathogenic bacteria database.

| Name                  | ST221_31B | SK222_32B | Gene Functiona l Annotation | Functional Annotation |
|-----------------------|-----------|-----------|-----------------------------|-----------------------|
| Vi antigen            | -         | -         | vexEDCBA/tviEDCBA            | Capsule               |
| Agf/Csg               | +         | +         | csgGFEDABC                  | Fimbrial adherence determinants |
| Bcf                   | +         | +         | bcfABCDEF                   | Fimbrial adherence determinants |
| Fim                   | +         | +         | fimAICDHFZYW                | Fimbrial adherence determinants |
| Lpf                   | +         | +         | lpfEDCBA                    | Fimbrial adherence determinants |
| Peg                   | -         | -         | pegDCBA                     | Fimbrial adherence determinants |
| Saf                   | +         | +         | safABCD                      | Fimbrial adherence determinants |
| SeF                   | -         | -         | selABCD                      | Fimbrial adherence determinants |
| Sta                   | -         | -         | staGFEDCBA                  | Fimbrial adherence determinants |
| Stb                   | +         | +         | stbABCDE                     | Fimbrial adherence determinants |
| Stc                   | +         | +         | stcDCBA                      | Fimbrial adherence determinants |
| Std                   | +         | +         | stdCBA                       | Fimbrial adherence determinants |
| Ste                   | -         | +         | steABCDEF                    | Fimbrial adherence determinants |
| Stf                   | +         | +         | stdACDEFG                    | Fimbrial adherence determinants |
| Stg                   | -         | -         | stgABCD                      | Fimbrial adherence determinants |
| Sth                   | +         | +         | sthEDCBA                     | Fimbrial adherence determinants |
| Sti                   | +         | +         | stiABCH                      | Fimbrial adherence determinants |
| Stk                   | +         | +         | stkBC                        | Fimbrial adherence determinants |
| Stj                   | -         | +         | stkGFEDCBA                   | Fimbrial adherence determinants |
| Tcf                   | -         | -         | tcfABCD                      | Fimbrial adherence determinants |
| Mig-14                | +         | +         | mig-14                       | Macrophage inducible gene |
| Mg2+ transport        | +         | +         | mgtBC                        | Magnesium uptake       |
| MisL                  | +         | +         | misLBC                       | Non fimbrial adherence determinants |
| RatB                  | +         | -         | ratB                         | Non fimbrial adherence determinants |
| ShdA                  | +         | +         | shdA                         | Non fimbrial adherence determinants |
| TTSS (SPI-1 encode)   | +         | +         | SPI-1                        | Secretion system       |
| TTSS (SPI-2 encode)   | +         | +         | TTSS                         | Secretion system       |
| TTSS effectors translocated via both systems | +         | +         | stlP                         | Secretion system       |
| TTSS-1 translocated effectors | +         | +         | sopB                         | Secretion system       |
| TTSS-1 translocated effectors | +         | +         | sopE2                        | Secretion system       |
| TTSS-1 translocated effectors | +         | +         | spoA                         | Secretion system       |
| TTSS-1 translocated effectors | +         | +         | avrA                         | Secretion system       |
| TTSS-1 translocated effectors | +         | +         | sptP                         | Secretion system       |
| TTSS-1 translocated effectors | +         | +         | sipA                         | Secretion system       |
| TTSS-1 translocated effectors | +         | +         | sipC                         | Secretion system       |
| TTSS-1 translocated effectors | +         | +         | sipB                         | Secretion system       |
| TTSS-1 translocated effectors | +         | +         | sopD                         | Secretion system       |
| TTSS-2 translocated effectors | +         | -         | ssel                         | Secretion system       |
| TTSS-2 translocated effectors | +         | +         | pipB                         | Secretion system       |
| TTSS-2 translocated effectors | +         | +         | silA                         | Secretion system       |
| TTSS-2 translocated effectors | +         | +         | ssaB                         | Secretion system       |
| TTSS-2 translocated effectors | +         | +         | sseF                         | Secretion system       |
| TTSS-2 translocated effectors | +         | +         | sseG                         | Secretion system       |
| TTSS-2 translocated effectors | +         | +         | stilB                        | Secretion system       |
| TTSS-2 translocated effectors | +         | +         | sseJ                         | Secretion system       |
| TTSS-2 translocated effectors | +         | -         | sseK2                        | Secretion system       |

(Continued)
Both serovars harbored genes for fimbrial adhesion and type III secretion system (TSS3). However, sseI, sseK2, ssph2, and sodCl genes were only present in ST221_31B. Of interest, T3SS-2 effector, SspH2, is conserved among most Salmonella serovars and translocated through T3SS2 (Type III secretion system 2) [34]. And, Salmonella Typhimurium periplasmic superoxide dismutase SodCl is encoded by the Gifsy-2 prophage. This is required for protection of non-typhoidal bacteremia associated strains of Salmonella against phagocyte oxidative burst [35].

**Phenotype characterizations by phenotype microarray**

Both Salmonella Typhimurium (ST221_31B) and S. Kentucky (SK222_32B) strains were characterized phenotypically using the OmniLog® PM system. Of the 960 conditions tested, only

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**Table 2. (Continued)**

| Name                      | ST221_31B | SK222_32B | Gene | Functional Annotation |
|---------------------------|-----------|-----------|------|-----------------------|
| TTSS-2 translocated effectors | +         | -         | ssph2 | Secretion system     |
| TTSS-2 translocated effectors | +         | +         | ssel  | Secretion system     |
| TTSS-2 translocated effectors | -         | -         | gogb  | Secretion system     |
| TTSS-2 translocated effectors | +         | +         | pipb2 | Secretion system     |
| TTSS-2 translocated effectors | +         | +         | ssek1 | Secretion system     |
| SodCl                     | +         | -         | sodcl | Stress protein        |
| Typhoid toxin             | -         | -         | cdtb/pltAB | Toxin       |
| PhoPQ                      | +         | +         | phoPQ | Two component system |

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**Table 3. Phenotypic microarray substrate utilization.**

| Substrate                         | ST221_31B | SK222_32B |
|-----------------------------------|-----------|-----------|
| 1,2-Propanediol                   | +         |           |
| α-Hydroxyglutaric Acid-γ-Lactone  | +         |           |
| L-Threonine                       | +         |           |
| α-D-Lactose                       | +         |           |
| m-Inositol                        | +         |           |
| N-Acetyl-D-Mannosamine            | +         |           |
| Adonitol                          | +         |           |
| Formic Acid                       | +         |           |
| Acetoacetic Acid                  | +         |           |
| α-Ketoglutaric Acid               | +         |           |
| D-Galactonic Acid-γ-Lactone       | +         |           |
| β-Methyl-D-Glucuronic Acid        | +         |           |
| D-Tagatose                        | +         |           |
| L-Tartaric Acid                   | +         |           |
| L-Cysteine                        | +         |           |
| Ammonia                           |           |           |
| O-Phospho-L-Threonine             |           |           |
| D,L-Ethionine                     |           |           |
| 60mM Sodium Nitrate               |           |           |
| 80mM Sodium Nitrate               |           |           |
| 100mM Sodium Nitrate              |           |           |
| pH 4.5                            |           |           |

*indicates significantly better utilization of the substrate based on area under the curve of the parametric data given by OmniLog® PM software.

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22 variable biochemical traits were identified. ST221_31B was found to utilize 14 carbon sources that strain SK222_32B did not; namely, 1,2-propanediol, α-hydroxyglutaric acid-g-lactone, L-threonine, α-D-lactose, β-methyl-D-glucuronic acid, M-inositol, N-acetyl-D-mannosamine, D-tagatose, adonitol, formic acid, acetoacetic acid, L-tartaric acid, α-ketoglutaric acid, and D-galactonic acid-g-lactone as well as one nitrogen source, L-cysteine. Conversely,
strain SK222_32B was found to be more resistant to certain stress conditions, as compared to ST221_31B ("Table 3").

Intracellular killing of Salmonella in macrophage

To evaluate the intracellular survival of ST221_31B and SK222_32B in macrophages, HD-11 and RAW 264.7 cell cultures in a standard gentamicin protection assay or macrophage killing assay were used [27]. "Fig 3" shows the CFU counts of viable intracellular Salmonella recovered from macrophage lysates obtained at 24 h and 48 h after infection of macrophages. Salmonella Typhimurium (ST221_31B) survival was significantly higher compared to S. Kentucky (SK222_32B). By 48 h, SK222_32B was almost completely eliminated while ST221_31B was reduced to approximately 10% of the CFU at time 0 h.

Invasion and growth of Salmonella in chicken ovarian granulosa cells

The invasiveness and replication potential of S. Typhimurium (ST221_31B) and S. Kentucky (SK222_32B) was evaluated in an ex vivo chicken ovarian granulosa cell model. As shown in "Fig 4", SK222_32B was unable to replicate in the granulosa cells while, a 3-fold growth of ST221_31B was observed by 24 h post infection.

Discussion

Despite extensive research efforts to characterize thousands of Salmonella strains every year by serotyping, pulsed-field gel electrophoresis (PFGE) fingerprinting, and antimicrobial resistance profiling by food safety stakeholders, food-borne S. enterica infection is still a major concern worldwide [36]. The genetic basis underlying the phenotypic diversity of S. enterica is not
This sentence is a paragraph of text that discusses the role of Salmonella enterica in disease.
we strived to corroborate genomic differences by demonstrating corresponding metabolic responses. Phenotype microarray results identified several substrates that *S. Typhimurium* ST221_31B was able to more effectively metabolize, as compared to *S. Kentucky* SK222_32B. For at least four of these substrates, namely D-tagatose, myo-inositol, sialic acid operon, and D-galactonate, genomic differences were also clearly seen.

Of the phenotypic differences observed, of particular interest from a virulence perspective are differences in utilizing 1, 2-propanediol, m-inositol, and N-Acetyl-D-Mannosamine substrates. *S. Typhimurium* ST221_31B was found to metabolize these substrates at a higher rate as compared to *S. Kentucky* SK222_32B. The substrate 1,2-propanediol is a major degradation product of the two commonly found sugars in plants, e.g., rhamnose and fucose. It serves as an important carbon source for organisms (i.e., *Salmonella*) that thrive under anaerobic conditions similar to that within mammalian large intestine [42–43]. The *pdu* operon codes for propanediol utilization in *Salmonella* [44]. Studies showed that the ability to degrade 1,2-propanediol has been directly linked to *Salmonella* virulence [45–47]. Although the exact mechanism relating to its virulence is unknown, deleting the *pdu* operon in some *Salmonella* strains resulted in the loss of virulence in those strains [45]. Comparative genomics indicates that the *pdu* operon is a core genome element, and phylogenetic evidence corroborates that it has evolved in a similar fashion to the whole genome of *Salmonella* serovars (“S5 Fig”). It is unclear if subtle SNP differences between serovars have resulted in loss of function or reduced function of this operon in strain SK222_32B. Another explanation could be the loss or modification of an unknown regulatory gene involved in controlling expression of this gene cluster. In contrast, comparative genomics revealed that the genome of strain SK222_32B was missing the myo-inositol utilization operon and a sialic acid utilization operon, which is presumed to account for differences in the utilization of M-inositol and N-Acetyl-D-Mannosamine, a precursor of sialic acid, respectively. We hypothesize that both phenotypic traits may be important in virulence, but mutagenesis-virulence model testing is needed to test this supposition.

The ability of a pathogen to survive phagocytosis and replicate within the phagosome is an important strategy to evade host defense mechanisms. Pathogenic *Salmonella* sp. have the ability to survive within host macrophages and this is an important aspect of their virulence [48]. In addition to macrophages, *Salmonella* Enteritidis has been reported to invade and replicate within the chicken granulosa ovarian cells [29], a model that has been used to study the egg-yolk contaminating capability. In this study, we applied both approaches and compared the invasion and intracellular survival of *S. Typhimurium* (ST221_31B) and *S. Kentucky* (SK222_32B) strains in macrophages, and in primary ovarian granulosa cells. The results of this study demonstrate that in both assays, *S. Typhimurium* ST221_31B was significantly more successful in terms of intracellular survival, with *S. Kentucky* SK222_32B more rapidly eliminated from macrophages and unable to invade and proliferate in ovarian granulosa cells. The inability to invade and replicate within the ovarian granulosa cells suggests a lack of potential for the *S. Kentucky* isolate to contaminate eggs, as compared to *S. Typhimurium*.

The superoxide dismutase enzyme (SodCI), which is known to play a vital role in *Salmonella* pathogenicity by intercepting reactive oxygen species produced by the host innate immune response [49], was only present in the genome of *S. Typhimurium* ST221_31B.

As mentioned above, one of the major steps in bacterial pathogenicity is host invasion. *S. Typhimurium* accomplishes this by sending a battery of proteins into host cell cytoplasm by way of type-III secretion systems [50–51]. One of these proteins is SopB, an inositol phosphatase, which is essential for promoting host cytoskeleton rearrangement and subsequent *Salmonella* internalization. M-inositol serves as the structural basis for many inositol phosphates including SopB [52]. Comparative genomics revealed that the gene coding for SopB protein were present in both ST221_31B and SK222_32B genomes; however, the myo-inositol
catabolic pathway is not present in the genome of SK222_32B. The ability to utilize M-inositol, as well as 1,2-propanediol, by ST221_31B, as compared to SK222_32B, is one possible factor which may partly indicate that S. Kentucky strain SK222_32B may be unable to cause human infections. Additionally, the superoxide dismutase enzyme (SodC1), which is known to play a vital role in Salmonella pathogenicity by intercepting reactive oxygen species produced by the host’ innate immune response [53], was only present in the genome of S. Typhimurium ST221_31B. More Salmonella strains of both Typhimurium and Kentucky serovars need to be tested for utilization of these compounds in future to confirm this trend.

In conclusion, the survival of S. Typhimurium ST221_31B within the macrophage cells even after 48 h and a 3-fold increase in growth in chicken ovarian granulosa cells demonstrate that S. Typhimurium ST221_31B has the potential to infect chickens, and subsequently can lead to human infections. We identified several differential traits between these two strains, which may account for these differences in the in vitro pathogenicity models used in this study. S. Typhimurium ST221_31B harbored three type III secretion system effector protein-encoding genes that were missing in SK222_32B, namely, seeI, sseK2, and sspH2. Genome sequencing and corresponding phenotypic microarray results indicated that S. Typhimurium strain ST221_31B has the ability to utilize three carbon compounds, myo-inositol, sialic acid, and 1,2-propanediol, which have been linked to virulence, significantly better than S. Kentucky strain SK222_32B. Although, in this study, we cannot definitively point to one or several factors as direct causes for the apparent difference in virulence potential between serovars S. Typhimurium and S. Kentucky, several factors were identified as candidates for further, detailed studies, which could directly test their effect on virulence in Salmonella enterica. Moreover, it is important to conduct research using multiple strains of each serovar as comparison of only two MDR genomes may not necessarily be representative of the entire strain population of a certain serovar.

Supporting information

S1 Fig. The rooted phylogenetic tree based on core genome of S. enterica (A) Maximum likelihood tree, (B) Maximum parsimony tree. (DOC)

S2 Fig. Genome comparison of Salmonella enterica serovar Typhimurium ST221_31B with 9 other Salmonella genomes. (DOC)

S3 Fig. Genome comparison of Salmonella enterica serovar Kentucky SK222_32B with 9 other Salmonella reference genomes. (DOC)

S4 Fig. (A) Phylogenetic reconstruction of homologous Incl1 plasmid of Salmonella enterica, (B) BLASTN comparison of homologous Salmonella enterica Incl1 class plasmids. (DOC)

S5 Fig. pdu operon tree. (DOC)

S1 Table. General feature of S. Typhimurium (ST221_31B) and S. Kentucky (SK222_32B) genomes. (DOC)

S2 Table. Identification of gaps or genetic differences in Salmonella Typhimurium. (DOC)
S3 Table. Identification of gaps or genetic differences in *Salmonella Kentucky*.

(DOC)

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