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Comparative Genomics Approaches to Understanding Virulence and Antimicrobial Resistance of *Salmonella* Typhimurium ST1539 Isolated from a Poultry Slaughterhouse in Korea^S

Eunsuk Kim[1], **Soyeon Park**[2], **Seongbeom Cho**[3], **Tae-Wook Hahn**[2*], **and Hyunjin Yoon**[1,4*]

[1]*Department of Molecular Science and Technology, Ajou University, Suwon 16499, Republic of Korea*

[2]*College of Veterinary Medicine and Institute of Veterinary Science, Kangwon National University, Chuncheon 24341, Republic of Korea*

[3]*Department of Veterinary Pathobiology and Preventive Medicine, Research Institute for Veterinary Science and College of Veterinary Medicine, Seoul National University, Seoul 08826, Republic of Korea*

[4]*Department of Applied Chemistry and Biological Engineering, Ajou University, Suwon 16499, Republic of Korea*

Non-typhoidal *Salmonella* (NTS) is one of the most frequent causes of bacterial foodborne illnesses. Considering that the main reservoir of NTS is the intestinal tract of livestock, foods of animal origin are regarded as the main vehicles of *Salmonella* infection. In particular, poultry colonized with *Salmonella* Typhimurium (*S.* Typhimurium), a dominant serotype responsible for human infections, do not exhibit overt signs and symptoms, thereby posing a potential health risk to humans. In this study, comparative genomics approaches were applied to two *S.* Typhimurium strains, ST1539 and ST1120, isolated from a duck slaughterhouse and a pig farm, respectively, to characterize their virulence and antimicrobial resistance-associated genomic determinants. ST1539 containing a chromosome (4,905,039 bp; 4,403 CDSs) and a plasmid (93,876 bp; 96 CDSs) was phylogenetically distinct from other *S.* Typhimurium strains such as ST1120 and LT2. Compared to the ST1120 genome (previously deposited in GenBank; CP021909.1 and CP021910.1), ST1539 possesses more virulence determinants, including ST64B prophage, plasmid *spv* operon encoding virulence factors, genes encoding SseJ effector, Rck invasin, and biofilm-forming factors (*bcf* operon and *pefAB*). In accordance with the *in silico* prediction, ST1539 exhibited higher cytotoxicity against epithelial cells, better survival inside macrophage cells, and faster mice-killing activity than ST1120. However, ST1539 showed less resistance against antibiotics than ST1120, which may be attributed to the multiple resistance-associated genes in the ST1120 chromosome. The accumulation of comparative genomics data on *S.* Typhimurium isolates from livestock would enrich our understanding of strategies *Salmonella* employs to adapt to diverse host animals.

Keywords: *Salmonella* Typhimurium, comparative genomics, virulence, antibiotic resistance

Introduction

Salmonellosis is a foodborne illness caused by non-typhoidal *Salmonella* (NTS) serotypes and typically induces a self-limiting gastroenteritis characterized by diarrhea, fever, and abdominal cramps within 4 and 72 h [1, 2]. However, an infection may be life-threatening due to bacteremia and extra-intestinal colonization in infants, the elderly, and immunocompromised patients [1, 2]. NTS colonizes animal and human intestines and is shed into soil and water through feces, circulating within agricultural ecosystems. In industrialized countries, human infections have been closely associated with contaminated foods of animal origin such as poultry, pork, and beef. Among more than 2,500 serotypes, *S.* Enteritidis and *S.* Typhimurium rank among the most notorious serovars responsible for human infections [3, 4]. Interestingly, *S.* Enteritidis is mainly isolated in poultry commodities, while *S.* Typhimurium is

associated with a wide variety of animal commodities such as poultry, pork, and beef [4]. Due to the intimate correlation between contaminated poultry and human infection, poultry has been regarded as a primary vehicle transmitting NTS to humans. An additional risk factor in terms of poultry hygiene is vertical and horizontal transmission of *S.* Enteritidis and *S.* Typhimurium without morbidity and mortality within the broiler production system [5], which impedes prompt action to prevent bacterial transmission to other healthy animals and food commodities.

In the context of extensive horizontal gene transfer events among diverse bacterial species, the emergence of NTS resistant to multiple antimicrobial agents in intensive animal farming poses a serious challenge to the treatment of severe bacterial infections. Antibiotics are used to treat clinical diseases but are routinely overused as low-cost substitutes for hygiene measures and as growth promoters in some countries [6]. Accumulating evidence linking antibiotics abuse in livestock with the emergence of bacterial resistance implicates a potential role of farm animals in the transmission of antibiotic resistance. Recent surveillance reports show that antibiotic-resistant NTS isolates are most frequently recovered from pigs and poultry, which rank first and second, respectively, in the global consumption of antibiotics per animal biomass [7, 8]. Furthermore, a high prevalence of resistance to ciprofloxacin and cephalosporins has been observed in *Salmonella* isolates from humans and poultry products in Korea and China, where these antibiotics are routinely used in large-scale intensive husbandry systems [9-11].

In our previous study, we demonstrated that *S.* Typhimurium ST1120 isolated from swine feces contains multiple genes associated with virulence and antibiotic resistance in its genome and exhibited substantial invasion and intracellular survival abilities when added to host cells [12]. In order to improve our understanding of the prevalence of *Salmonella* in diverse livestock, *S.* Typhimurium ST1539 isolated from a duck slaughterhouse in Korea was subjected to whole genome sequencing, and its genetic loci associated with virulence and resistance were identified and compared with those of ST1120 in this study. *S.* Typhimurium ST1539, in agreement with the results of comparative genomic analysis, was more virulent than ST1120 in animal models but was more susceptible to antibiotics. Comparative genomics approaches to diverse *Salmonella* serotypes isolated from different livestock might provide insights into the mechanism of *Salmonella* adaptation in different host animals, as well as into the correlation between *Salmonella* serotypes and host preference.

Materials and Methods

Bacterial Strains and Growth Conditions

S. Typhimurium ST1539 was isolated from a duck slaughterhouse located in Yangpyeong, Gyeong-gi Province in Korea. *S.* Typhimurium ST1120 previously reported in our study was isolated from feces of pig [12]. All *S.* Typhimurium strains including LT2 [13], 14028s [14], and SL1344 [15] were grown in Luria Bertani (LB) broth (Difco, USA) or Mueller Hinton broth (Difco) at 37°C with shaking at 220 rpm.

Genome Sequencing and Assembly

S. Typhimurium ST1539 was cultivated in LB broth overnight. Bacterial cells were harvested by centrifugation at 16,000 ×*g* for 1 min and subjected to genomic DNA extraction using a GeneElute Bacterial Genomic DNA Kit (Sigma-Aldrich, USA). DNA concentration was estimated using a SimpliNano spectrophotometer (GE Healthcare, USA). A total of 5 µg of isolated DNA was used for SMRT sequencing (Pacific Biosciences, USA), conducted at LabGenomics Inc. (Korea). A total of 1,644,445,258 bp after sequencing was subjected to *de novo* assembly using the hierarchical genome assembly process (HGAP, Version 2.3) workflow [16] with 214 folds of genome coverage. Finally, the circular forms of contigs were checked using MUMmer 3.5 [17] and self-similar ends were trimmed for manual genome closure.

Genome Annotation and Analysis

For gene annotation, acquired contigs were processed using Prokka version 1.12 [18], GeneMark [19], and NCBI BLASTP [20]. RNAmmer 1.2 [21] and tRNAscan-SE [22] were used for rRNA and tRNA gene predictions, respectively. Analyzed contigs were deposited in GenBank under accession numbers CP035301 (chromosome) and CP035302 (plasmid). The genome was mapped using DNAPlotter [23], including prophage regions identified using PHAge Search Tool (PHAST) [24]. A genome tree was created using JSpeciesWS based on average nucleotide identity (ANI) [25]. *Salmonella in silico* typing resource (SISTR) [26] was used to predict the serotype of ST1539. BL2SEQ [27] was used to perform comparative analysis and the result was visualized using Easyfig [28]. Antibiotic resistance-associated genes were predicted using the Comprehensive Antibiotic Resistance Database (CARD) [29]. Virulence-associated genes were analyzed using SPIFinder server ver. 1.0 [30] and the Virulence Factors Database (VFDB) [31].

Pulsed-Field Gel Electrophoresis (PFGE) Analysis

PFGE analysis was conducted according to the methods of Tenover *et al*. [32] and Wonderling *et al*. [33] with slight modifications. *Salmonella* cells embedded in agarose plugs were prepared as described in the previous study [34]. *Salmonella* colonies were collected from LB agar plates and suspended in TE suspension buffer (100 mM Tris and 100 mM EDTA, pH 7.5). The turbidity of the bacterial cell suspension was adjusted to 20% transmittance using a colorimeter (bioMérieux, France). The cell

suspension was mixed with proteinase K and 1.2% agarose (FMC Bioproducts, USA), and then dispensed into disposable plug molds (Bio-Rad, USA) containing ES buffer (0.5 M EDTA, pH 9.0; 1% sodium-lauroyl-sarcosine) and proteinase K. The plugs were incubated in a water bath at 55°C for 1 h and then washed using sterile water and TE buffer (10 mM Tris and 1 mM EDTA, pH 7.5) preheated to 50°C. The washed plugs were cut into two 1-mm-wide slices and then incubated with XbaI (Promega, USA) at 37°C for 3 h. Digested DNAs in the plugs were separated using 1% agarose gel in the Contour Clamped Homogenous Field (CHEF) DR II electrophoresis system (Bio-Rad) at 14°C. *Salmonella* serotype Braenderup H9812 (ATCC, USA) restricted with XbaI was used as the universal size standard. After electrophoresis, the gel stained using 50 mg/ml ethidium bromide was observed on a UV trans-illuminator. Macro-restriction patterns were analyzed using BioNumerics software (Applied-Maths, Sin-Martens-Latem, Belgium) based on Dice coefficients with a 1% band position tolerance.

Antibiotic Susceptibility Test

Disk diffusion assay was applied to test antibiotic susceptibility [35]. Briefly, *Salmonella* cells were cultured in Mueller Hinton broth at 37°C up to the McFarland turbidity standard of 0.5. Sterile cotton swabs were used to spread bacterial cells evenly on Mueller Hinton agar plates. Disks containing antibiotics at indicated concentrations were placed onto the agar plates and incubated at 35°C for 16 to 18 h. The diameter of the growth inhibition zone was measured and used to interpret resistance or susceptibility according to the CLSI standards [36].

Lactate Dehydrogenase (LDH) Cytotoxicity Assay

HeLa cells (ATCC) were seeded onto 96-well cell culture plates at a density of 1×10^4 cells per well and incubated in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) prior to bacterial infection. *Salmonella* cells cultivated in LB broth overnight were washed and resuspended in DMEM broth, and then added to HeLa cells at a multiplicity of infection (MOI) of 100. At 6 h post-infection, bacterial cytotoxicity was evaluated using CytoTox-ONE Homogeneous Membrane Integrity Assay (Promega) according to the manufacturer's instructions as described in the previous study [12]. LDH released from damaged HeLa cells converts lactate to pyruvate and produces NADH, which in turn converts resazurin to the fluorescent compound resorufin. Fluorescence was measured using a fluorometer (BioTek Synergy HTX Multi-Mod Reader, USA). Intact HeLa cells not treated with *Salmonella* cells were used in parallel as a negative control.

Invasion and Survival Assays

Gentamycin protection assay was conducted to evaluate the ability of *Salmonella* to invade host cells and to survive inside host cells [12]. For the invasion assay, epithelial HeLa cells were seeded onto 24-well cell culture plates at a density of 1×10^5 cells per well containing DMEM supplemented with 10% FBS prior to

bacterial infection. *Salmonella* strains cultured in LB broth for 3 h were washed and resuspended in DMEM and added to HeLa cells at a MOI of 100. At 30 min post-infection, the medium was replaced with fresh DMEM containing gentamicin at 100 μg/ml to inactivate extracellular bacteria, and infected cells were further incubated for 1.5 h. HeLa cells were then lysed with 1% Triton X-100 (Sigma-Aldrich), and the lysates were diluted and plated on LB agar plates in order to count the intracellular bacteria. For survival assay, macrophage-like RAW264.7 (ATCC) cells were seeded onto 24-well cell culture plates at a density of 2×10^5 cells per well, and *Salmonella* cells at stationary phase in LB broth were added to host cells at a MOI of 100 and incubated for 30 min. After 1.5 h incubation with gentamicin (100 μg/ml) as described above, host cells were replenished with fresh DMEM containing gentamicin at 20 μg/ml for 9 h. Intracellular *Salmonella* cells were counted in the same way as described in the invasion assay.

Mouse Infection Experiment

For mouse infection experiments, 7-week-old BALB/c female mice were used according to protocols approved by the Kangwon University Institute Animal Care and Use Committee (Permit number: KW-160201-1). A total of 24 mice were divided into 6 groups and infected intraperitoneally with 1×10^2, 1×10^3, or 1×10^4 CFU of ST1539 or ST1120, respectively. Infected mice were monitored for two weeks and then euthanized according to the approved protocol.

Statistical Analysis

Every test was repeated at least three times using different bacterial colonies. Results were averaged and presented with their standard deviations. For statistics, Student's *t*-test was applied and *p*-values less than 0.05 were considered statistically significant.

Results

Understanding General Genome Characteristics of *S. Typhimurium* ST1539

Whole genome sequencing of *S.* Typhimurium ST1539 revealed two contigs constituting a circular chromosome of 4,905,039 bp and a plasmid of 93,876 bp. The chromosome was predicted to contain a total of 4,327 ORFs including 4,219 coding sequences (CDSs), 22 rRNA genes, and 86 tRNA genes with a 52.15% GC content, whereas 96 ORFs estimated in the plasmid were all predicted as CDSs with a 53.11% GC content (Fig. 1). In view of genetic exchanges through bacteriophages, *in silico* PHAST analysis [24] of ST1539 genome sequences identified 6 prophages including PaV-LD, RE-2010, Gifsy-1, ST64B, Gifsy-2, and Tyrion (Fig. 1), three of which (PaV-LD, RE-2010, and Tyrion) are rarely detected in *S.* Typhimurium strains [37, 38]. In compliance with the presence of distinct prophages in the
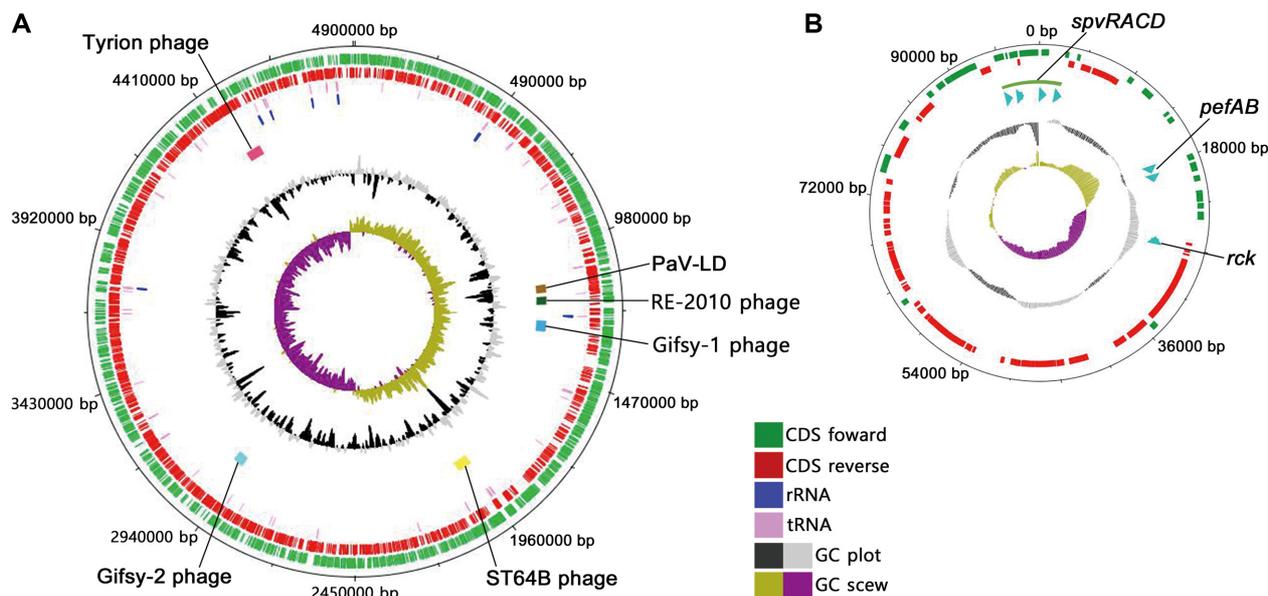
Fig. 1. Genome maps of *S.* Typhimurium ST1539.
The ST1539 genome that consists of the chromosome (A; 4,905,039 bp) and plasmid pST1539 (B; 93,876 bp) was mapped using DNAPlotter.

ST1539 genome, the phylogenetic tree analysis using ANI values proposed that ST1539 was not intimately associated with other *S.* Typhimurium strains, though it still belonged to a cluster of *S.* Typhimurium strains in comparison with other serotypes (Fig. 2). Serotyping of ST1539 determined it to be Typhimurium by a serological test based on Kauffmann-White scheme [39] and via *in silico* prediction using SISTR [26]. ST1539 also showed a distinct XbaI-PFGE pattern from other *S.* Typhimurium strains such as LT2 and 14028s in PFGE analysis (Fig. S1). ST1539 was fused with other *S.* Typhimurium strains at a higher distance of 79.4% in a dendrogram calculated with Dice coefficients, while ST1120 had a 95.7% sequence similarity with LT2. ST1120 reported in our previous study (GenBank Acc. No.
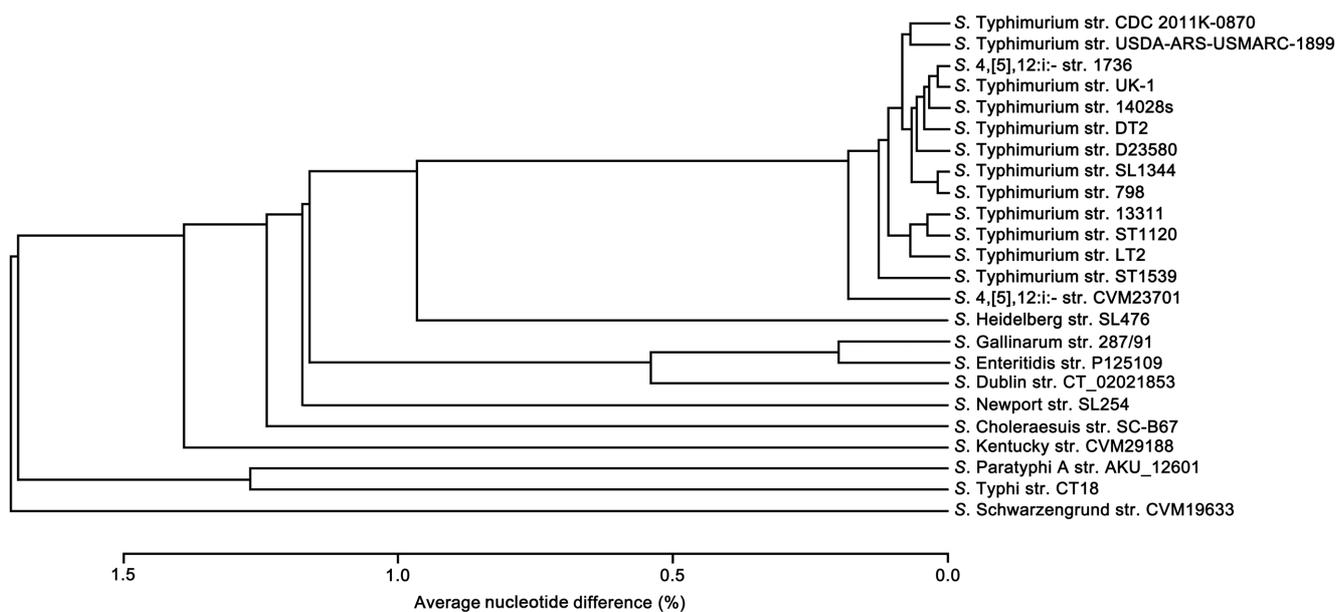
Fig. 2. Phylogenetic tree analysis of *S.* Typhimurium ST1539.
The genome sequence of ST1539 was compared to those of other *Salmonella* serotypes by ANI using JSpeciesWS.

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CP021909.1 and CP021910.1; [12]) is a *S.* Typhimurium strain isolated from swine feces in Korea. In an effort to understand the strategies of *S.* Typhimurium in adapting to diverse livestock, the strains ST1539 and ST1120 with a genetic divergence in the serogroup Typhimurium were further investigated in terms of their genetic determinants associated with virulence and antibiotic resistance.

Evaluation of Antibiotic Resistance of *S.* Typhimurium ST1539

In our previous study, we showed that ST1120 was more resistant to several antibiotics such as streptomycin, chloramphenicol, and ampicillin than other *S.* Typhimurium strains LT2, 14028s, and SL1344, and we observed a large number of genes in its genome that were presumably

responsible for the resistance properties [12]. Considering that piggery and poultry farms are the top 2 in antibiotic use in animal farming, ST1539 was likely to develop tolerance against antibiotics extensively used in poultry farming. However, the *in silico* comparative analysis with ST1120 using CARD predicted that ST1539 lacked multiple genes encoding components of efflux pumps, which play an important role in conferring resistance by actively excreting the harmful antibiotic drugs from the bacteria (Table 1). When the two isolates were treated with 10 different antibiotics, ST1539 exhibited susceptibility to all the tested antibiotics according to the CLSI standards and was more susceptible than ST1120 specifically to gentamicin, kanamycin, and neomycin (Fig. 3 and Table S1). ST1120 isolated from a pig farm was somewhat resistant to six

Table 1. CDCs predicted to be associated with antimicrobial resistance.

ARO[a] category	ST1539 Locus_tag	ST1120 Locus_tag
Efflux pump complex or subunit conferring antibiotic resistance	ST1539_0378, ST1539_1019, ST1539_1021, ST1539_1807, ST1539_1918, ST1539_2111, ST1539_2877, ST1539_2953, ST1539_3114, ST1539_3335, ST1539_3336, ST1539_3456, ST1539_3458, ST1539_3459, ST1539_3460, ST1539_4395	ST1120_01136, ST1120_01137, ST1120_02874, ST1120_02875, ST1120_00207, ST1120_02873, ST1120_02870, ST1120_01647, ST1120_00208, ST1120_01138, ST1120_02872, ST1120_04131, ST1120_04132, ST1120_01684, ST1120_01685, ST1120_03550, ST1120_03551, ST1120_03958, ST1120_00647, ST1120_02167, ST1120_04203, ST1120_01279, ST1120_01724, ST1120_04342, ST1120_00707, ST1120_01277, ST1120_02263, ST1120_01899, ST1120_03223, ST1120_01140, ST1120_03925, ST1120_02871, ST1120_01810, ST1120_01911, ST1120_02695, ST1120_03549, ST1120_00711, ST1120_00978, ST1120_01366, ST1120_02503, ST1120_00010, ST1120_02757, ST1120_02428, ST1120_03007, ST1120_04535, ST1120_01260, ST1120_01261
Aminocoumarin resistance	N/D	ST1120_02466, ST1120_03558
Aminoglycoside resistance	ST1539_2243	ST1120_01481, ST1120_02368
Beta-lactam resistance	N/D	ST1120_02584, ST1120_02215
Cephamycin resistance	ST1539_3699	N/D
Elfamycin resistance	ST1539_0399, ST1539_4317	N/D
Fluoroquinolone resistance	ST1539_2345, ST1539_2346, ST1539_4143, ST1539_4144	ST1120_01961
Fosfomycin resistance	ST1539_1539	N/D
Isoniazid resistance	N/D	ST1120_00256
Mupirocin resistance	N/D	ST1120_00771
Nitrofuran resistance	ST1539_2946	N/D
Peptide antibiotic resistance	N/D	ST1120_03945
Polymyxin resistance	N/D	ST1120_02823, ST1120_03041, ST1120_03042, ST1120_00428
Sulfonamide resistance	N/D	ST1120_00839

[a]ARO (antibiotic resistance ontology) analyzed by Resistance Gene Identifier (RGI) according to CARD (comprehensive antibiotic resistance database).
N/D, Not detected.

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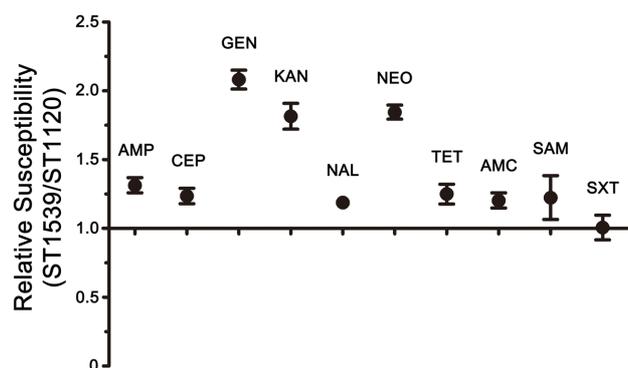
Fig. 3. Comparison of antibiotic resistance profiles between ST1539 and ST1120.

Antibiotic susceptibility test of ST1539 and ST1120 was conducted on Mueller Hinton agar plates using antibiotic disks. Diameters of growth inhibition zones were measured and used to calculate relative susceptibility between ST1539 and ST1120. The ratios from three independent assays were averaged and plotted. The antibiotics tested were AMP, ampicillin; CEP, cephalothin; GEN, gentamicin; KAN, kanamycin; NAL, naladixic acid; NEO, neomycin; TET, tetracycline; AMC, amoxicillin/clavulanic acid; SAM, ampicillin/sulbactam; SXT, sulfamethoxazole/trimethoprim. The concentration of each antibiotic was determined according to the CLSI standards for testing against Enterobacteriaceae family.

antibiotics and especially exhibited higher resistance to a class of aminoglycosides including gentamicin, kanamycin, and neomycin, which reflects the overuse of aminoglycoside antibiotics in pig farms ever since the mid-1980s in Korea [40, 41]. In the case of β-lactam antibiotics, ST1120 showed an intermediate level of resistance against ampicillin, but

both isolates ST1120 and ST1539 were determined to be susceptible to the combinations (amoxicillin/clavulanic acid and ampicillin/sulbactam) supplemented with β-lactamase inhibitors. Taken together, the pig farm isolate ST1120 was likely to develop resistance against β-lactams and aminoglycosides due to frequent exposure to these antibiotics. According to a recent surveillance on antibiotic resistance in poultry commodities in Korea, *Salmonella* isolates from duck meat showed much lower resistance to a variety of antibiotics, including ampicillin, tetracycline, and nalidixic acid, than those from chicken meat [42], which can be attributed to varying antibiotic usage across poultry species. A statistical analysis using a large number of *Salmonella* isolates would define the correlation between antibiotic consumption levels and antibiotic resistance prevalence in livestock.

Exploring Virulence Determinants in *S.* Typhimurium ST1539 Genome

The potential virulence of ST1539 was assessed by searching for virulence-associated determinants in the genome (Table 2). *Salmonella* pathogenicity islands (SPIs) are the best known genetic loci critical for *Salmonella* virulence. Virulence effectors encoded by SPIs are translocated to the cytosol of host cells through bacterial type III secretion systems (T3SSs) and manipulate host cellular functions for bacterial invasion and proliferation inside hosts [43, 44]. Eight clusters of SPI-1, SPI-2, SPI-3, SPI-4, SPI-5, SPI-12, SPI-13, and SPI-14 were all found in both ST1539 and ST1120 (Table 2).

Bacteriophage-mediated horizontal gene transfer diversifies
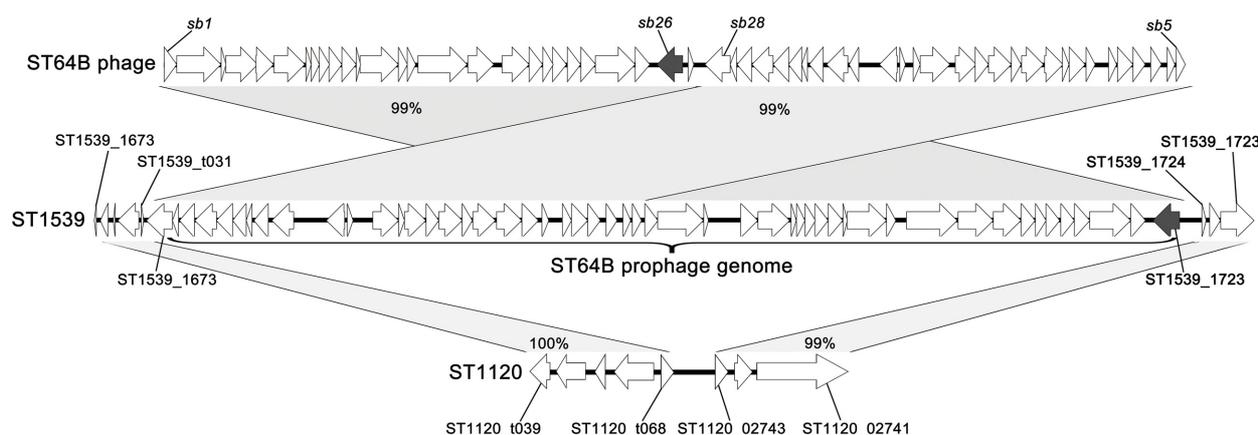
Fig. 4. Sequence alignment of ST64B phage with ST539 and ST1120.

The ST64B prophage region of *S.* Typhimurium ST1539 (middle) was compared with those of ST64B phage (GenBank Acc. No. AY055382; top) and *S.* Typhimurium ST1120 (bottom) using Easyfig. Gene *sb26* and its homolog ST1539_1723 are indicated with grey arrows within the phage ST64B (top) and prophage ST64B (middle), respectively.

bacterial genomic repertoires and enables them to adapt to environmental changes efficiently during host infections by integrating virulence-associated genes into the genome [45, 46]. In comparison with other *S.* Typhimurium strains such as LT2 and ST1120, ST1539 possesses the prophage ST64B, which was first identified in *S.* Typhimurium DT 64 (Fig. 4) [47]. ST64B prophage covers a 40,149-bp region containing 51 CDSs from ST1539_1673 to ST1539_1723, and its role

Table 2. Virulence factors *in silico* predicted in ST1539 and ST1120.

Virulence factor	ST1539 Locus tag (gene)	ST1120 Locus tag (gene)	Function
[a,†]SPI-1	ST1539_0869 (*invH*) - ST1539_0901 (*avrA*)	ST1120_03592 (*avrA*) - ST1120_03632 (*invH*)	*Salmonella* pathogenicity island 1
[a,†]SPI-2	ST1539_2241 (*ssaU*) - ST1539_2271 (*ssrB*)	ST1120_02123 (*ssrB*) - ST1120_02166 (*ssaU*)	*Salmonella* pathogenicity island 2
[a,†]SPI-3	ST1539_0074 (*mgtC*) - ST1539_0085	ST1120_04484 - ST1120_04497 (*mgtC*)	*Salmonella* pathogenicity island 3
[a,†]SPI-4	ST1539_3811 (*siiF*) - ST1539_3815 (*siiA*)	ST1120_00391 (*siiA*) - ST1120_00398 (*siiF*)	*Salmonella* pathogenicity island 4
[a,†]SPI-5	ST1539_2542 (*copR*) - ST1539_2549 (*pipA*)	ST1120_01833 (*pipA*) - ST1120_01841 (*copR*)	*Salmonella* pathogenicity island 5
[a,†]SPI-12	ST1539_1454 (*pagL*) - ST1539_1465	ST1120_02972 - ST1120_02988 (*pagL*)	*Salmonella* pathogenicity island 12
[a,†]SPI-13	ST1539_0658 (*exuT*) - ST1539_0674	ST1120_03852 - ST1120_03871 (*exuT*)	*Salmonella* pathogenicity island 13
[a,†]SPI-14	ST1539_2724 - ST1539_2731	ST1120_01634 - ST1120_01640	*Salmonella* pathogenicity island 14
[a,†]C63PI	ST1539_1265 - ST1539_1271	ST1120_03251 - ST1120_03258	Centisome 63 pathogenicity island
[a†] effectors	ST1539_0828 (*sopD*), ST1539_1018 (*pipB2*), ST1539_1202 (*sseB*), ST1539_1204 (*gogB*), ST1539_1476 (*sseL*), ST1539_1520 (*sspH1*), ST1539_1618 (*sseK2*), ST1539_1685 (*sopA*), ST1539_1723 (*sseK3*), ST1539_2064 (*steC*), ST1539_2090 (*steA*), ST1539_2135 (*sseJ*), ST1539_2164 (*sifB*), ST1539_2306 (*steB*), ST1539_2532 (*sifA*), ST1539_2660 (*sopD2*), ST1539_2903 (*slrP*), ST1539_2903 (*slrP*), ST1539_4130 (*sseK1*)	ST1120_03677 (*sopD*), ST1120_03515 (*pipB2*), ST1120_03273(*sseb*), ST1120_03322 (*gogB*), ST1120_03030 (*sseL*), ST1120_02984 (*sspH1*), ST1120_02880 (*sseK2*), ST1120_02809 (*sopA*), N/D, ST1120_02449 (*steC*), ST1120_02332 (*steA*), N/D, ST1120_02351 (*sifB*), ST1120_02378 (*steB*), ST1120_01970 (*sifA*), ST1120_01712 (*sopD2*), ST1120_01575 (*slrP*), ST1120_01575 (*slrP*), ST1120_00294 (*sseK1*)	T3SS effectors
[b,†]*lpf* operon	ST1539_0209 (*lpfA*) - ST1539_0214 (*lpfE*)	ST1120_04370 (*lpfE*) - ST1120_04374 (*lpfA*)	Long polar fimbriae
[b,†]*csg* operon	ST1539_2605 (*csgC*) - ST1539_2611 (*csgG*)	ST1120_01884 (*csgG*) - ST1120_01890 (*csgC*)	Curli fimbriae
[b,†]*fim* operon	ST1539_3130 (*fimF*) - ST1539_3134 (*fimI*)	ST1120_01329 (*fimI*) - ST1120_01333 (*fimF*)	Type 1 fimbriae
[b,†]*bcf* operon	ST1539_3641 (*bcfG*) - ST1539_3647 (*bcfA*)	N/D	Fimbriae
[b,†]*mig-14*	ST1539_1017	ST1120_03517	Antimicrobial peptide resistance protein
[b,†]*ompA*	ST1539_2677	ST1120_01817	Outer membrane protein A
[a,‡]*spv* operon	ST1539_p001 (*spvC*), ST1539_p002 (*spvD*), ST1539_p091 (*spvR*), ST1539_p093 (*spvA*)	N/D	Plasmid encoded virulence proteins
[a,‡]*pef* operon	ST1539_p017(*pefA*), ST1539_p018(*pefB*)	N/D	Plasmid encoded fimbriae
[a,‡]*rck*	ST1539_p025	N/D	Plasmid encoded invasin

[a]Identified by SPIFinder ver.1.0.

[b]Identified by Virulence Factors DataBase.

[†]Chromosome of ST1539, ST1120.

[‡]Plasmid of ST1539.

N/D, Not detected.

associated with *S.* Typhimurium fitness has been demonstrated in *S.* Typhimurium isolates from human blood samples [48]. The prophage region was strongly conserved in *S.* Typhimurium strains isolated from blood samples and its presence favored bacterial growth in human blood and plasma. SseK3 encoded by *sb26* (homologous to ST1539_1723) within the phage ST64B is a member of SseK/NleB effector proteins. Its expression is co-regulated with other SPI-2 genes by SsrB, a primary transcriptional regulator of SPI-2, and SseK3 is translocated into the host cytosol by SPI-2 T3SS [49].

Aside from the ST64B prophage, ST1539 encompasses multiple virulence-relevant determinants encoding SseJ effector, SpvC/SpvD effectors, fimbriae (*bcf* operon and *pefAB*), and Rck invasin (Table 2). Some of these genes including *spvCD*, *pefAB*, and *rck* are uniquely located in the

plasmid of ST1539 (Fig. 1 and Table 2).

Assessment of Virulence of *S.* Typhimurium ST1539 In Vitro and In Vivo

Comparative genomic analysis between ST1539 and ST1120 predicted more virulence-associated genetic features in ST1539 (Table 2). We previously observed that ST1120 was competent to invade into host epithelial cells and to replicate inside macrophages when compared with other virulent *S.* Typhimurium strains such as 14028s and SL1344 [12]. The virulence of ST1539 was compared with that of ST1120 to verify the *in silico* prediction. When epithelial HeLa cells were treated with *S.* Typhimurium strains (LT2, 14028s, SL1344, ST1120, and ST1539), ST1539 had the highest cytotoxic activity to the host cells (Fig. 5A), whereas all strains except LT2 showed comparable invasion ability
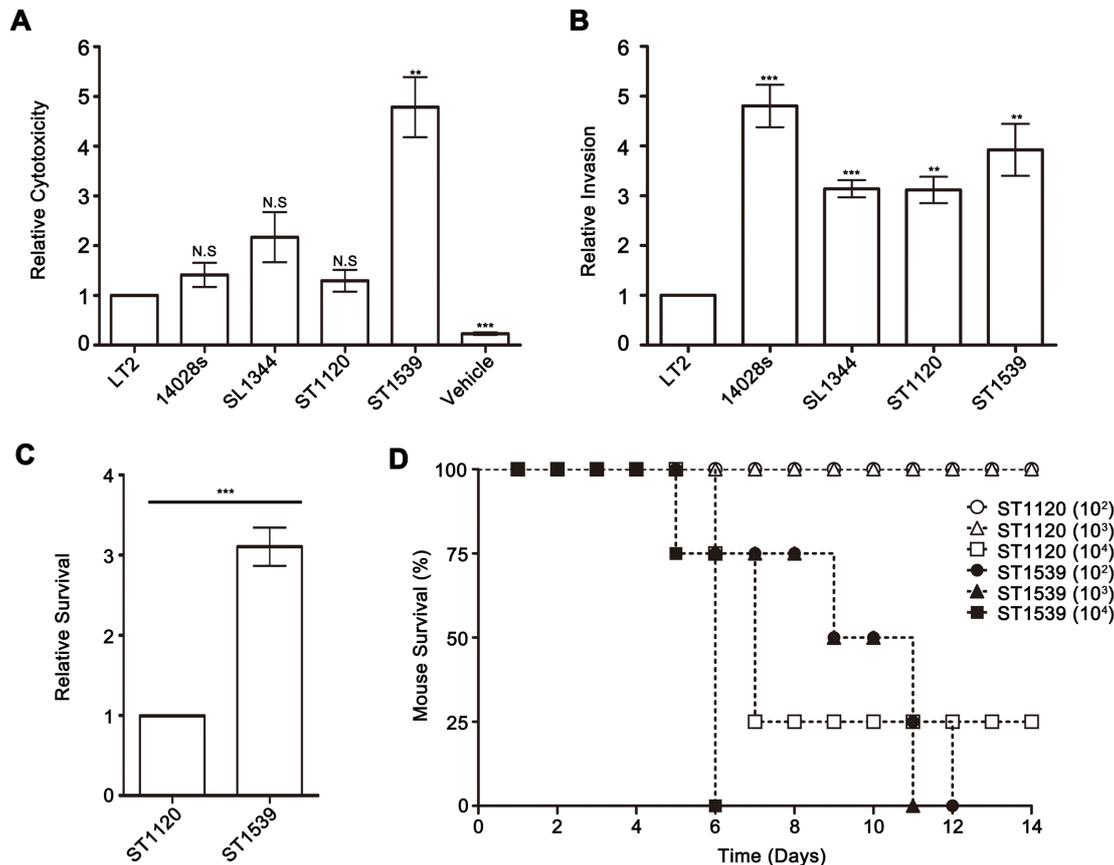
Fig. 5. Virulence comparison in vitro and in vivo between ST1539 and ST1120.
Cytotoxicity (**A**) and invasion ability (**B**) of ST1539 were compared with those of other *S.* Typhimurium strains (LT2, 14028s, SL1344, and ST1120) using epithelial HeLa cells. The levels of cytotoxicity and invasion ability of each strain were compared with those of LT2 and the ratios were plotted. Differences with *p*-values less than 0.05 (**) or 0.01 (***) in comparison with LT2 were denoted with asterisks. N.S. indicates no significance in comparison with the control. Survival ability between ST1539 and ST1120 was compared using macrophage-like RAW264.7 cells (**C**). Asterisks indicate a difference of *p*-value less than 0.01 between the two strains. For animal tests (**D**), BALB/c mice were intraperitoneally infected with ST1539 and ST1120 at different doses (10^2, 10^3, and 10^4 CFU/mouse) and their survival rates were plotted for 2 weeks.

(Fig. 5B). The attenuated virulence of LT2 has been previously reported *in vitro* and *in vivo* elsewhere [50, 51]. In the survival assay using RAW264.7 macrophage-like cells, ST1539 replicated faster than ST1120 inside host cells, showing 1.5-fold higher cell numbers at 9 h post-infection (Fig. 5C), which might be attributable to multiple virulence determinants present in ST1539. The ability to persist and replicate inside macrophages is crucial in developing systemic infections in host animals [52]. We further compared the ability to conquer host animals between ST1539 and ST1120 (Fig. 5D). Mice intraperitoneally infected with ST1120 at 10^3 CFU/mouse survived during 14 days of observation but ST1539 killed all mice even at a lower dose of 10^2 CFU/mouse, indicating its hypervirulence *in vivo*. These results are consistent with the *in silico* analysis results demonstrating multiple virulence attributes in the ST1539 genome.

In summary, we aimed to characterize the properties of ST1539 in the context of antimicrobial resistance and virulence based on the genome sequences. Its resistance and virulence were compared with those of ST1120, which was also isolated from livestock in Korea. Data about the genome properties of *Salmonella* isolates would provide insights into the evolutionary adaptation process of *Salmonella* during environmental changes in the livestock industry, as well as help find a way to manage *Salmonella* infections transmitted via contaminated foods of animal origin.

Acknowledgments

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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