speG Is Required for Intracellular Replication of Salmonella in Various Human Cells and Affects Its Polyamine Metabolism and Global Transcriptomes

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The speG gene has been reported to regulate polyamine metabolism in Escherichia coli and Shigella, but its role in Salmonella remains unknown. Our preliminary studies have revealed that speG widely affects the transcriptomes of infected in vitro M and Caco-2 cells and that it is required for the intracellular replication of Salmonella enterica serovar Typhimurium (S. Typhimurium) in HeLa cells. In this study, we demonstrated that speG plays a time-dependent and cell type-independent role in the intracellular replication of S. Typhimurium. Moreover, high-performance liquid chromatography (HPLC) of four major polyamines demonstrated putrescine, spermine, and cadaverine as the leading polyamines in S. Typhimurium. The deletion of speG significantly increased the levels of the three polyamines in intracellular S. Typhimurium, suggesting the inhibitory effect of speG on the biosynthesis of these polyamines. The deletion of speG was associated with elevated levels of these polyamines in the attenuated intracellular replication of S. Typhimurium in host cells. This result was subsequently validated by the dose-dependent suppression of intracellular proliferation after the addition of the polyamines. Furthermore, our RNA transcriptome analysis of S. Typhimurium SL1344 and its speG mutant outside and inside Caco-2 cells revealed that speG regulates the genes associated with flagellar biosynthesis, fimbrial expression, and functions of types III and I secretion systems. speG also affects the expression of genes that have been rarely reported to correlate with polyamine metabolism in Salmonella, including those associated with the periplasmic nitrate reductase system, glucarate metabolism, the phosphotransferase system, cytochromes, and the succinate reductase complex in S. Typhimurium in the mid-log growth phase, as well as those in the ilv–leu and histidine biosynthesis operons.
INTRODUCTION

Non-typhoidal Salmonella are important pathogens that cause a wide spectrum of diseases and considerable morbidity and mortality in humans and animals worldwide (Hohmann, 2001). Salmonella are invading and intracellularly replicating bacteria (Dougan et al., 2011). Host cell invasion (Pace et al., 1993) and intracellular replication (Leung and Finlay, 1991) are essential for the pathogenesis of Salmonella enterica serovar Typhimurium (S. Typhimurium). More than 100 virulence-associated genes have been discovered among the approximately 4,500 genes present in the genome of S. Typhimurium (McClelland et al., 2001). Most virulence genes are clustered in at least 23 Salmonella pathogenicity islands (SPIs) distributed on the Salmonella chromosome (Espinoza et al., 2017), including 11 common SPIs in S. Typhimurium and S. Typhi (Sabbagh et al., 2010). The most commonly studied SPIs, SPI-1 and SPI-2, encode type III secretion systems (T3SSs), which can translocate effector proteins into host cells or secrete them into the extracellular environment to manipulate host cell physiology and biochemistry (Coburn et al., 2007). SPI-1 genes facilitate bacterial invasion in non-phagocytic cells and uptake into phagocytic cells in the early phase of infection (Bueno et al., 2010). By contrast, SPI-2 genes account for intracellular survival and the evasion of the oxidative defense system of host cells, particularly in the systemic phase of salmonellosis (Coburn et al., 2007). The SPI-2 T3SS is essential for bacterial intracellular replication in Salmonella-containing vacuoles in host cells through the translocation of approximately 30 SPI-2 T3SS effector proteins into the host endomembrane system and cytosol (Figueira and Holden, 2012). However, the physiological relevance of SPI-2 T3SS effectors and the effects of their coordination on SPI-2 T3SS-mediated intracellular replication remain unclear (Helaine et al., 2010). Until now, SPI-2 T3SS genes associated with the intracellular replication of Salmonella have been mostly reported in phagocytic cells (Helaine et al., 2010; Figueira and Holden, 2012; Figueira et al., 2013). A few studies have demonstrated that SPI-1 T3SS genes are required for intracellular replication in human cervical epithelial cells (Steele-Mortimer et al., 2002) and 3-dimensional colonic epithelial cells (Radtke et al., 2010). Furthermore, additional studies have reported that auxotrophic mutations in aromatic amino acid metabolism and purine biosynthesis attenuated the intracellular replication of S. Typhimurium in various cell lines, including Madin–Darby canine kidney epithelial cells, human cervical HeLa cells, and intestinal epithelial Caco-2 and T-84 cells (Leung and Finlay, 1991; Holzer and Hensel, 2012). However, virulence genes involved in the intracellular replication of Salmonella in human non-phagocytic epithelial cells have not been thoroughly investigated.

Following the isolation of 10 auxotrophic replication-defective mutants from the 45,000 transposon mutants of S. Typhimurium in 1991 (Leung and Finlay, 1991), large-scale screening studies using high-throughput technologies, including libraries of transposon mutants and transcriptomic analysis, have identified previously unreported genes that are required for intracellular replication in non-phagocytic cells. The intracellular proliferation of S. Typhimurium occurs in cultured epithelial and macrophage cells but not in normal fibroblast cells (Martinez-Moya et al., 1998; Cano et al., 2001; Nunez-Hernandez et al., 2013). Thus, 50,000 independent transposon MudJ-generated mutants derived from wild-type S. Typhimurium were selected in rat kidney fibroblasts after 72-h intracellular incubation. Genome analysis of the non-proliferating intracellular mutants revealed that a novel gene, igA, suppresses their growth within fibroblasts (Cano et al., 2001). Meanwhile, mutations in phoQ, rpoS, slyA, and spvR, which have been demonstrated to be essential for the in vivo intracellular proliferation of Salmonella, resulted in the attenuation of intracellular bacterial growth in fibroblasts. This suggested that the PhoP–PhoQ two-component system is a negative regulator of bacterial growth in fibroblasts and so are the different phenotypes of these genes in diverse cell types (Cano et al., 2001). A recent genome-wide study conducted using the expression profiling of non-growing wild-type S. Typhimurium collected at 24 h postinfection in the same rat fibroblasts revealed that approximately 2% of the S. Typhimurium genome was differentially expressed in non-proliferating intracellular bacteria. This included the 98 genes involved in metabolic reprogramming for microaerophilic conditions, the induction of virulence plasmid genes, the upregulation of SPI-1 and SPI-2, and the shutdown of chemotaxis and flagellation (Nunez-Hernandez et al., 2013). Similarly, the transcriptome showed activated functions of PhoP–PhoQ-regulating PagN, PagP, and VirK in dormant intracellular bacteria after sensing vacuolar acidic pH for preventing intracellular overgrowth (Nunez-Hernandez et al., 2013). Another non-phagocytic cell line, HeLa cell line, has been extensively used to study intracellular replication of not
only *Salmonella* spp. but also of enteroinvasive *Escherichia coli* (*E. coli*) and *Yersinia* spp. (Small et al., 1987; Leung and Finlay, 1991; Hautefort et al., 2008). The microarray analysis of time-dependent changes in *Salmonella* gene expression in HeLa cells and J774A.1 murine macrophages demonstrated the upregulation of *iro, mgtBC*, and *pstACS*; genes for iron, magnesium, and phosphate uptake; and SPI-2 (Hautefort et al., 2008). The invasion-associated SPI-1 and flagellar genes are upregulated in epithelial cells at 6h postinfection when bacteria are intracellularly replicating but are constantly downregulated in J774A.1 murine macrophages (Hautefort et al., 2008). A recent study conducted using a mutational approach reported that the replication of *S. Typhimurium* in murine colonic epithelial cells requires glycosis and ubiquinone, but not an intact tricarboxylic acid cycle (TCA cycle), adenosine triphosphate (ATP) synthase, and fermentation (Garcia-Gutierrez et al., 2016). It remains unclear whether malate could be replenished by succinate or its precursors from non-phagocytic cells similar to phagocytes, although conversion from succinate to fumarate, from fumarate to malate, and from malate to both oxaloacetate and pyruvate in the TCA cycle are required for full virulence of *S. Typhimurium* in mice (Tchawa Yimga et al., 2006; Mercado-Lubo et al., 2008, 2009). So far, the virulence genes involved in the intracellular replication of *Salmonella* in human intestinal epithelial cells have not been thoroughly investigated.

Our preliminary study conducted using a library of 1,440 transposon mutants of *S. Typhimurium* to invade HeLa cell monolayers for 10h and a high-throughput genome-wide analysis through transposon-directed insertion-site sequencing (Chaudhuri et al., 2013) identified *speG* as a gene essential for the intracellular replication of *Salmonella* in human epithelial cells (Fang, 2011). However, it remains unknown whether this result is applicable to other cells. The *speG* mutant of *S. Typhimurium* is a non-replicating strain in human cells and is thus a candidate vaccine vector for interacting with intestinal epithelial cells (Wang et al., 2016b). We used RNA microarrays to determine whether *S. Typhimurium* and *speG* affect the transcriptomes of two human intestinal epithelial cells and identified *speG*-regulated genes, including *KY14, SCTR, IL6, TNF*, and *CELF4* in Caco-2 cells and *JUN, KLF6*, and *KCTD11* in *in vitro* M cells, which are specialized intestinal epithelial cells conferring host immunity (Wang et al., 2016b). However, it is unclear whether *speG* regulates the expression of other genes in *Salmonella* before and after bacterial invasion in human intestinal epithelium.

Until now, knowledge regarding *speG* has been obtained from studies mainly conducted in *E. coli* and *Shigella*, but rarely in *Salmonella*. *speG* is involved in polyamine metabolism and stress responses in bacterial pathogenesis. It encodes spermidine acetyltransferase (SAT), which catalyzes spermidine to acetyl spermidine in *E. coli*. However, the *speG*-dependent acetylation of spermidine and the *speE*-dependent catabolization of cadaverine into aminopropyl cadaverine are not conserved in *Shigella* spp. (Barbagallo et al., 2011). The accumulation of spermidine is toxic for *E. coli* and reduces the viability of the *speG*-deficient mutant of *E. coli* at the late stationary growth phase. However, excessive spermidine can be inactivated by its *speG*-catalyzed acetylation to acetylspermidine, which is either stored or secreted by the cells (Fukuchi et al., 1995). The other *spe* genes, including *speB, speC, speE*, and *speF*, have been reported to contribute to the intracellular survival and replication of *S. Typhimurium* in human epithelial cells for 18h (Jelsbak et al., 2012) and in macrophages for 21h (Espinell et al., 2016). However, the role of *speG* in regulating polyamine metabolism and influencing the intracellular replication of *Salmonella* in human intestinal epithelial cells has been rarely investigated.

Polyamine composition and the predominant polyamine in *Salmonella* are unclear. Until now, our understanding of polyamines in bacteria has mainly been established through studies of *E. coli*. Putrescine, spermidine, spermine, and cadaverine are the major cellular polyamines essential for the normal cellular proliferation and growth of both prokaryotic and eukaryotic cells (Cohen, 1997; Shah and Swiatlo, 2008). The intracellular concentration of spermidine is much higher than that of putrescine in almost all bacteria, but 10 times lower than that of putrescine in *E. coli* (Cohen, 1997; Shah and Swiatlo, 2008). Spermine is only found in the presence of exogenous spermine in most bacteria, whereas cadaverine, typically absent in *E. coli*, is the least widespread of naturally occurring bacterial polyamines (Cohen, 1997). Putrescine constitutes the outer membrane of *S. Typhimurium* and *E. coli* (Koski and Vaara, 1991). However, whether putrescine is the predominant intracellular polyamine in *Salmonella*, similar to *E. coli*, requires further validation.

In this study, we examined whether the deletion of *speG* affects the intracellular replication of *S. Typhimurium* in various human cells. Subsequently, we studied the polyamine metabolism of *S. Typhimurium* and the effect of *speG* by quantifying the four major polyamines in extracellular and intracellular wild-type and *speG*-deleted strains. We verified whether the accumulation of polyamines suppresses the intracellular proliferation of *S. Typhimurium*. Moreover, we investigated how *speG* regulates the transcriptome of *S. Typhimurium* before and after invasion in human intestinal epithelium. Finally, we determined whether the deletion of *speG* affects the motility and flagellation of *S. Typhimurium*.

### MATERIALS AND METHODS

#### Bacterial Strains and Culture Conditions

The *S. Typhimurium* wild-type strain SL1344, its isogenic *speG*-deleted mutant ΔspeG, the *speG*-complemented strain of ΔspeG (*ΔspeG*), the *fliC*-deleted flagellin-deficient mutant Δ*fliC*, and *spaS*-deleted invasion-deficient SPI-1 mutant Δ*spaS* were used in this study. SL1344 (Mo et al., 2006) and Δ*spaS* (Buckley et al., 2010) were kindly provided by Prof. Duncan Maskell. The *S. Typhimurium* SL1344 genome has been completely sequenced, and its complete sequence and annotation are available in Genbank (accession numbers FQ312003 and HE654724-6). The mutants Δ*fliC* and Δ*spaS* were used as controls. Δ*speG* and Δ*fliC* were constructed using the lambda red recombinase-mediated integration of linear polymerase chain reaction (PCR) amplicons to replace the target gene with a kanamycin resistance gene cassette, as previously reported (Gust et al., 2003; Wang et al., 2016a,b). For generating the
speG-complemented S. Typhimurium strain (ΔspeG), the speG-coding sequence was amplified using speG-specific primers (forward, 5'-ATCTTACTGGCCTGTTGCTAGT-3', and reverse, 5'-GATGACGATAACTAAAAGAAGTGTAAAGGATCGT-3') and cloned into the pBluescript II KS(−) vector in the EcoRV site. Subsequently, the cloned vector was digested by EcoRV and ligated with the apramycin resistance gene, aac(3)IV, which was then amplified using the aac(3)IV specific primers (forward, 5'-TACCAACCTGTAGTACGGTGTT-3', and reverse, 5'-AAACCGGGCGGGGTGCTACTCTCGTGACTACCCGCCGCGACGCTGATCGTGCGGGAG-3'), then transferred into the ΔspeG mutant. The speG expression was restored following the same red recombination strategy as previously prescribed (Wang et al., 2016a). The ΔspeG was maintained in LB broth supplemented with apramycin (50 µg/mL) at 37°C. This study was conducted in the Biosafety Level 2 Laboratory that had been approved by the Biosafety Committee of Taipei Medical University Shuang Ho Hospital (No. BSL-2-0001).

In Vitro Cell Cultures

Four human cell lines used in this study were purchased from Bioresource Collection and Research Center, Taiwan. HeLa cells (BCRC No. 60005, originally from ATCC CCL-2), which are an epithelial cell line of human cervical carcinoma, and LS174T cells (BCRC No. 60053, originally derived from ATCC CL-188), which are a human intestinal epithelial cell line of Caucasian Duke's type B colorectal adenocarcinoma, were cultured in 90% Dulbecco's modified Eagle medium (DMEM, 4,500 mg/L glucose; Gibco) complemented with 2 mM L-glutamine (Gibco) adjusted to contain 1.5 g/L sodium bicarbonate (Sigma), 0.1 mM non-essential amino acids (Gibco), 1 mM sodium pyruvate (Gibco), and 10% fetal bovine serum (FBS, Sigma). Furthermore, Caco-2 cells (BCRC No. 67001, originally from ATCC HTB-37), a human intestinal epithelial cell line of a Caucasian colon adenocarcinoma, were cultured in the same medium as that for HeLa cells, except for the substitution of 10% FBS with 20% FBS. THP-1 cells (BCRC No. 60430, originally from ATCC TIB-202), a cell line of human acute monocytic leukemia, were cultured in a suspension of 90% RPMI 1640 medium (Gibco) complemented with 2 mM L-glutamine (Gibco) adjusted to contain 1.5 g/L sodium bicarbonate, 2.5 g/L glucose, 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol (Gibco), and 10% FBS. For maintenance, these cells were grown in 75-cm² flasks in humidified 5% CO₂ at 37°C and were split in a 1:4 ratio of 0.25% trypsin–ethylenediaminetetra acetic acid (Gibco) before complete confluence.

For in vitro infection assays, these cells were seeded at a density of 5 × 10⁵ cells/well into 12-well plates and were maintained in humidified 5% CO₂ at 37°C. The cell culture medium was replaced every other day when the cells were incubated for 3, 4, and 5 days until a complete confluence of the cell monolayers was achieved at a density of approximately 1 × 10⁶ cells/well for HeLa cells, 8 × 10⁵ cells/well for Caco-2 cells, and 2 × 10⁶ cells/well for LS 174T cells, respectively. Meanwhile, THP-1 cells in suspension were seeded at a density of 2 × 10⁵ cells/well into 12-well plates and were incubated with 10 ng/well phorbol myristate acetate (Sigma) for 24 h to induce differentiation into adherent macrophages for further assays. The medium in each well of the cell monolayer was replaced with their corresponding complete medium without FBS 1 h before assays.

Bacterial Intracellular Replication Assay

HeLa cell monolayers were infected with overnight cultures of S. Typhimurium SL1344, ΔspeG, and ΔspeG [multiplicity of infection (MOI) = 5] in duplicate wells for each bacterial strain and were incubated in 5% CO₂ at 37°C for 2 h. After washing three times with phosphate-buffered saline (PBS), the infected cells were incubated in PBS-free DMEM supplemented with gentamicin (100 µg/mL) for 1 h; thereafter, the cells were washed with PBS three times to kill extracellular bacteria. At this point, one set of the cells infected with the three S. Typhimurium strains was lysed with 1% Triton X-100 to generate output pool A, which represents invading bacteria. The other two sets of the cells infected with the S. Typhimurium strains were incubated for an additional 7 and 10 h in FBS-free DMEM supplemented with low-dose gentamicin (10 µg/mL) to allow intracellular infections to continue. The cells were subsequently washed with PBS three times and lysed with 1% Triton X-100 (Sigma) to generate output pools B1 and B2, respectively, which represent intracellularly replicating bacteria after incubation at different durations.

The prepared confluent HeLa, Caco-2, LS 174T, and THP-1 cells in 12-well plates were infected with overnight cultures of the three S. Typhimurium strains (MOI = 5) for 2 h and treated with gentamicin (100 µg/mL) for 1 h using the same protocol as that used for obtaining output pool A. After washing with PBS three times, the cells were incubated for an additional 15 h. Finally, the cells were lysed with 1% Triton X-100 to generate output pool B, which contained bacteria proliferated in the cells for a total of 18 h.

The infected cell monolayers were stained with trypan blue to confirm a viability of >95% in all the wells before cell lysis to obtain output pools B1, B2, and B. The intracellular bacterial counts respectively to the initial inoculums in output pools A, B1, B2, and B were compared between the two recombinant strains and wild-type strain of S. Typhimurium by using the Student's t-test. The intracellular bacterial counts in all output pools are expressed as mean ± standard error colony-forming units (CFU) per inoculum of 10⁷ CFU. p < 0.05 was considered statistically significant.

High-Performance Liquid Chromatography Quantification of Four Major Polyamines in S. Typhimurium SL1344 and ΔspeG before and after Invasion in Caco-2 Cells

Before the experiment, overnight cultures of S. Typhimurium SL1344 and ΔspeG were 1:100 diluted in Luria–Bertani (LB)
broth and incubated with shaking at 225 rpm in 5% CO₂ at 37°C for 3 h to generate mid-log cultures, which were considered as extracellular bacteria. The prepared confluent Caco-2 cells in the 75-cm² flasks were infected with mid-logarithmic cultures of S. Typhimurium SL1344 and ΔspeG (MOI = 5) for a total of 18 h by using the same protocol as that for obtaining the output pool B in three independent experiments. The Caco-2 cells infected by these two strains of S. Typhimurium were lysed with 1% Triton X-100 to obtain the intracellular bacteria.

Next, polyamines in extracellular bacteria from the mid-log cultures of S. Typhimurium SL1344 and ΔspeG, and the Caco-2 cell lysates containing intracellular bacteria of these two strains were extracted using trichloroacetic acid (TCA; Sigma). The mid-log cultures were centrifuged (4,000 ×g) at 4°C for 10 min; the supernatants were removed, and the bacterial pellets were washed with PBS. The centrifugation and PBS washing protocols were performed twice, and the bacterial pellets were resuspended in lysis buffer [20 mM 3-(N-morpholino) propanesulfonic acid, pH 8.0, 10 mM NaCl, and 4 mM MgCl₂]. The bacterial cells were lysed through ultrasonic vibration. Finally, 100 µL of 40% TCA was added to the bacterial lysates on ice for 5 min and centrifuged (13,000 ×g) at 4°C for 3 min. The supernatants of both extracellular S. Typhimurium strains were decanted and stored at −20°C for high-performance liquid chromatography (HPLC) analysis.

The intracellular bacteria in the Caco-2 cell lysates were filtered using a 7-µm filter to remove the cell debris, and the pellets were resuspended in LB broth under shaking at 225 rpm in 5% CO₂ at 37°C for 2 h to amplify the bacterial concentration of the two host cell-prime S. Typhimurium strains. The bacterial pellets were subsequently processed as TCA precipitation and polyamine extraction for the mid-log cultures of both S. Typhimurium strains. Finally, the supernatants of both intracellular strains were collected and stored at −20°C for HPLC.

The standard solutions of putrescine (purity: 99.9%; TCI), spermidine (purity: 99.7%; Fluka), spermine (purity: 99.8%; Fluka), and cadaverine (purity: 98.7%; 1,5-diaminopentane, Fluka) were 1:4 diluted and mixed for HPLC analysis to obtain the retention time of putrescine at 35 min, cadaverine at 40 min, spermidine at 54 min, and spermine at 65 min (Supplementary Figure 1). Next, the five dilutions of the four polyamine standard solutions and the four TCA-treated bacterial samples were filtered using Hypersil ODS C18 Columns (Thermo Scientific) and injected into the HPLC apparatus (Waters 600 controller). Subsequently, the peak area values of the four polyamines in the bacterial samples and the serial dilutions of the standard solutions in the HPLC chromatogram were obtained and analyzed using the Autochro-3000 Chromatography Data System (Young Lin, Taiwan). Finally, the concentrations of the four polyamines in the TCA-treated bacterial samples were calculated by applying their peak area values to the regression equations derived from the five dilutions of the analyzed standard solutions (Supplementary Figure 2). These samples were bracketed with standards in five dilutions, including putrescine (1, 2, 5, 10, and 20 mM; peak areas between 40 and 1,200 mm²), cadaverine (0.1, 0.2, 0.5, 1, and 2 mM; peak areas between 100 and 3,500 mm²), spermidine (0.1, 0.2, 0.5, 1, and 2 mM; peak areas between 50 and 2,500 mm²), and spermine (1, 2, 5, 10, and 20 mM; peak areas between 40 and 1,500 mm²). The concentrations of the individual polyamines were compared between S. Typhimurium SL1344 and ΔspeG before and after their invasion in Caco-2 cells by using the Student's t-test. Similarly, the polyamine concentrations of extracellular and intracellular bacteria were also compared in S. Typhimurium SL1344 and ΔspeG, respectively. The polyamine concentrations are expressed as mean ± standard error (mM per 10⁸ bacteria). p < 0.05 was considered statistically significant.

Polyamine Suppression Assay

By using the same protocol as that used for obtaining output pool B, confluent Caco-2 cells in 12-well plates were infected with overnight cultures of S. Typhimurium SL1344, and the infected cells were treated with putrescine (625 and 312.5 µM), spermine (375 and 187.5 µM), and cadaverine (125 and 62.5 µM) or left untreated for 15 h. After 18-h incubation, the intracellular bacterial numbers from the treated Caco-2 cells were calculated as prescribed in the bacterial intracellular replication assays and compared between the treated and untreated groups by using the Student's t-test. The data are expressed as mean ± standard error CFU per inoculum of 10⁷ CFU. p < 0.05 was considered statistically significant.

RNA Microarrays of S. Typhimurium SL1344 and ΔspeG before and after Invasion in Caco-2 Cells

Confluent Caco-2 cells in the 75-cm² flasks were infected with overnight cultures of S. Typhimurium SL1344 or ΔspeG (MOI = 5) in two independent experiments by using the same protocol as that used for obtaining the output pool B. After 18-h
incubation, the infected cells were lysed with 1% Triton X-100, and the cell lysates were passed through a 3-μm filter to remove the cell debris. After centrifugation at 800 × g for 10 min and the removal of supernatants, the bacterial pellets were washed with PBS to obtain the intracellular bacteria. The extracellular bacteria from the overnight cultures and the intracellular bacteria from the aforementioned processing of S. Typhimurium SL1344 and ΔspeG were dissolved in TRIzol (Gibco) for isolating the total RNA according to the manufacturer's instruction. The purity of the RNA samples was validated using the ratio of absorbance at 260 and 280 nm, as well as the RNA integrity number determined using Bioanalyzer 2100 (Agilent Technology) with an RNA 6000 Nano LabChip kit (Agilent).

*In vitro* transcription was performed as previously described (Lee et al., 2009). Briefly, the total RNA samples were reverse transcribed to cDNAs and subsequent cRNAs, which were amplified and labeled with Cy3 (CyDye, Agilent). The Cy3-labeled cRNAs were subsequently fragmented to an average size of 50–100 nucleotides, pooled, and hybridized to Agilent Technologies custom Salmonella GE 8 × 15K microarray that had been tiled with 4,631 gene probes of S. Typhimurium SL1344. After washing the array chips and drying them through nitrogen gun blowing, the microarrays were scanned with an Agilent microarray scanner at 535 nm for Cy3-Cy5. The scanned images were quantified and analyzed using Feature Extraction 10.5.1.1 software (Agilent). The background values were corrected using the spatial detrend surface value and were normalized by quantile. Finally, the gene expression in each array group was analyzed using the DAVID database (https://david.ncifcrf.gov/).

A heap map with genes in each group that showed more than two-fold upregulation or downregulation was constructed based on their normalized values by using GeneSpring multiomic analysis software (Agilent). The microarray data has been deposited in GEO (http://www.ncbi.nlm.nih.gov/geo/) and is accessible via the GEO Accession Number GSE102885.

The transcriptomes derived from S. Typhimurium SL1344 and ΔspeG were compared before and after their invasion to Caco-2 cells by using the Student's t-test. The data were expressed as mean ± standard error log2 fold change relative to S. Typhimurium SL1344. p < 0.05 was considered statistically significant.

### Quantitative Real-Time Polymerase Chain Reaction for Confirmation of RNA Analysis
Pairs of oligonucleotide primers specific to the selected genes identified from the microarrays and the housekeeping gene 16S (Table 1) were designed using Primer3 and BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The total RNAs of extracellular and intracellular S. Typhimurium SL1344 and ΔspeG were isolated from their mid-log cultures and lysates of the infected Caco-2 cells by using the Total RNA Miniprep Purification Kit (Genemark, Taichung, Taiwan) according to the manufacturer's instruction. The total RNA samples were purified, and the residual DNA was eliminated using RNase-free DNase I (NEB, Beverly, MA, USA). Next, 0.1 μg of RNA was reverse transcribed to cDNA by using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany), according to the manufacturer's instruction. By using the Bio-Rad C100 Real-Time PCR System, quantitative real-time PCR (qRT-PCR) was performed in triplicate in a reaction volume of 25-μL solution containing 0.2 μM of primer pairs, 12.5 μL of iQ SyBr green supermix (BioRad), 9.5 μL of distilled H2O, and 1 μL of cDNA. The reaction solutions were heated at 95°C for 3 min and amplified for 40 cycles of 95°C for 15 s, 50°C for 30 s, and 72°C for 30 s. The mRNA transcription levels were determined using the ΔΔCt method, as previously described (Wang et al., 2016b), and the expression of 16s ribosomal RNA was considered for normalization. The mRNA expression of the selected genes in extracellular and intracellular S. Typhimurium ΔspeG mutant was compared with that of the same genes in the corresponding extracellular and intracellular S. Typhimurium SL1344 by using the Student's t-test. The data were expressed as mean ± standard error log2 fold change relative to S. Typhimurium SL1344. p < 0.05 was considered statistically significant.

### Bacterial Motility Assays
Semisolid LB agar plates containing 0.3% agar were used for performing the bacterial motility assay. Ten microliters of overnight cultures of S. Typhimurium SL1344, ΔspeG, ΔspeG′, ΔspaS, and ΔflIC were inoculated on the centers of the semisolid LB agar plates, followed by incubation at 37°C for 6 h. In this assay, the flagellated non-invasive SPI-1 mutant S. Typhimurium ΔspaS was considered the positive control, whereas the flagella-deficient mutants S. Typhimurium ΔflIC was used as the negative control. Furthermore, 10 μL of overnight cultures of S. Typhimurium SL1344 were inoculated on the centers of the semisolid LB agar plates supplemented with putrescine (625 and 312.5 μM), spermine (375 and 187.5 μM), cadaverine (125 and 62.5 μM), spermidine (6 and 3 μM), or left without any polyamine, and all the plates were incubated at 37°C for 6 h. The intensity of bacterial motility was determined by the diameters of bacterial growth zones. All the above assays were performed in three independent experiments.

### Transmission Electron Microscopy
S. Typhimurium SL1344 and ΔspeG were visualized through transmission electron microscopy after negative staining. The overnight bacterial cultures were centrifuged at 5,000 rpm for 20 min, washed with distilled water twice, and fixed with 4% paraformaldehyde for 10 min. The bacteria on the grids were washed with distilled water twice, negatively stained with 2% uranyl acetate for 30 s, and subsequently rinsed with distilled water thrice. Finally, the samples were observed under a transmission electron microscope (FEI Tecnai G2 F20 S-Twin FEG).

### RESULTS

**speG Affects the Intracellular Replication of S. Typhimurium in Human Cell Lines**

To confirm whether speG affects the intracellular proliferation of S. Typhimurium in infected human epithelial cells with time,
HeLa cells were infected with S. Typhimurium SL1344 and ΔspeG for 10, 13, and 18 h after killing extracellular bacteria during the third hour postinfection by performing gentamicin protection assays. Although S. Typhimurium ΔspeG showed non-significant attenuation in bacterial proliferation in HeLa cells after 10 and 13 h postinfection (Figure 1A), intracellular S. Typhimurium ΔspeG was significantly attenuated in output pool B in HeLa cells at 18 h postinfection, but not in output pool A at 3 h postinfection (1.1 × 10^7 vs. 1.9 × 10^7 CFU/well, p < 0.05; Figure 1B). Moreover, to investigate whether such a phenomenon existed in different cell types, the same in vitro intracellular replication assays were performed in Caco-2, LS174T, and THP-1 cells. We observed that the intracellular concentrations of S. Typhimurium ΔspeG significantly decreased in LS174T cells (1.9 × 10^7 vs. 2.1 × 10^7 CFU/well, p < 0.05; Figure 1D) and THP-1 cells (1.0 × 10^7 vs. 1.3 × 10^7 CFU/well, p < 0.05; Figure 1E) compared with those of S. Typhimurium SL1344. The results revealed that S. Typhimurium SL1344 and ΔspeG invaded HeLa, LS174T, and THP-1 cells in similar concentrations, but the deletion of speG significantly attenuated the intracellular concentration of S. Typhimurium in these three cell lines after 18 h. Intracellular S. Typhimurium ΔspeG significantly attenuated in both output pools A and B in Caco-2 cells (3.5 × 10^5 vs. 2.4 × 10^5 CFU/well and 1.3 × 10^6 vs. 8.8 × 10^5 CFU/well, respectively, p < 0.05; Figure 1C), with significantly higher attenuation of S. Typhimurium ΔspeG than of S. Typhimurium SL1344 in output pool B than in output pool A (9.6 × 10^5 vs. 6.4 × 10^5 CFU/well, p < 0.05; Figure 1C). This suggested that the knockout of speG decelerated the intracellular amplification of S. Typhimurium in Caco-2 cells.

| Primer name (F: forward, R: reverse) | Sequence (5′ → 3′) | Product size (base pairs) | Description |
|-------------------------------------|--------------------|---------------------------|-------------|
| fha-A-F                             | TGTAGTGCGGCCGCTTACCT | 124                       | Flagellar gene |
| fha-A-R                             | GAAGCGGCAGCAAGATCCA | 124                       | Flagellar gene |
| fha-B-F                             | GTTCGGCGGCCGATGTTAG  | 175                       | Flagellar gene |
| fha-B-R                             | AAGGCACCAAGACACAG   | 175                       | Flagellar gene |
| flp-F                               | TTCTCTGGCGGCTGTGCGG | 149                       | Flagellar gene |
| flp-R                               | TCAGGCGGCAAAGGTCAGC | 149                       | Flagellar gene |
| flq-F                               | TGATGGTGGAAGGACAGC  | 183                       | Flagellar gene |
| flq-R                               | ACGGGGCAGCACGATAATT | 183                       | Flagellar gene |
| fgl-F                               | ACCAGAGAAGCGAGCGCG  | 160                       | Flagellar gene |
| fgl-R                               | ATGCAGTGCTTGGTGCGG  | 160                       | Flagellar gene |
| flgH-F                              | AGATGGCGCCTGGATACCGG | 183                       | Flagellar gene |
| flgH-R                              | CAATGCGGACGGTACGCCG | 183                       | Flagellar gene |
| fmc-F                               | TGAGCAGCAGACGACCCCT | 183                       | Type-1 fimbrial gene |
| fmc-R                               | CGGTTGCCGGCTATGAG    | 183                       | Type-1 fimbrial gene |
| fmd-F                               | ACCCTGCGGGCTGTGCGG  | 186                       | Type-1 fimbrial gene |
| fmd-R                               | GCAATGGCGGGCCGGTTTAC | 186                       | Type-1 fimbrial gene |
| fim-F                               | TGCGAGCAGCAGTATGCTG  | 149                       | Type-1 fimbrial gene |
| fim-R                               | CGAAACTGCGAAGCCGCTG  | 149                       | Type-1 fimbrial gene |
| fimW-F                              | TCGCGCGAATCAGTAACCG  | 149                       | Type-1 fimbrial gene |
| fimW-R                              | GCACCTGCGGCGCCGCGATTA | 186                       | Type-1 fimbrial gene |
| ilvC-F                              | AAATGCGGCGAGGAGT     | 243                       | ilv−leu operon |
| ilvC-R                              | CGCAAGCAGTAGAGAACAG  | 243                       | ilv−leu operon |
| leuD-F                              | GCTCTGCGGACATCTTCA   | 159                       | ilv−leu operon |
| leuD-R                              | TATCGCGGCTTGATCCAC   | 159                       | ilv−leu operon |
| hisG-F                              | GTCTGGTAAATGATGGCT   | 237                       | Histidine operon |
| hisG-R                              | GTAGAGGCGAAGAGT      | 237                       | Histidine operon |
| SL1344_2430-F                       | CGTCGGCGCATGATTCTC   | 153                       | Putative cobalamin adenosyltransferase |
| SL1344_2430-R                       | ATCGCGAGTTTTACACC    | 153                       | Putative cobalamin adenosyltransferase |
| smv-A-F                             | TCTCAGCGCGTCTATCCGCT | 221                       | Methyl viologen resistance protein |
| smv-A-R                             | GCTAGACACATCTAGCT    | 221                       | Methyl viologen resistance protein |
| pyrE-F                              | AACGCGAAGAGGCGAAAAAG | 163                       | Orotate phosphoribosyltransferase |
| pyrE-R                              | TCAAGTACGCGCGAGAGT   | 163                       | Orotate phosphoribosyltransferase |
| speG-F                              | GTAATTACGATATGTACCG  | 149                       | Spermidine N1-acetyltransferase |
| speG-R                              | CGTTACTGGTTGAAGGCC   | 149                       | Spermidine N1-acetyltransferase |
| 16S-F                               | TCCTCGACATTGGCGGCA   | 190                       | Housekeeping gene 16S |
| 16S-R                               | TTCTCTGGCGGCTGGAAC   | 190                       | Housekeeping gene 16S |
FIGURE 1 | Intracellular bacterial replication assays yielded the intracellular bacterial concentrations of S. Typhimurium SL1344 and its ΔspeG mutant at various time points after invasion in different human cell lines. Human cell lines were infected with S. Typhimurium wild-type SL1344, its ΔspeG mutant, and the speG-complemented strain ΔspeG′ (multiplicity of infection = 5) for 1 h. The extracellular bacteria were killed using gentamicin after another 2 h, and the intracellular bacteria in the infected cells were allowed to proliferate for additional 7 h (output pool B1), 10 h (output pool B2), or 15 h (output pool B). (A) S. Typhimurium ΔspeG was non-significantly attenuated in output pools B1 and B2, in which intracellular bacteria in HeLa cells had been maintained for 10 and 13 h, respectively. (B–E) S. Typhimurium ΔspeG was significantly attenuated in output pool B, in which intracellular bacteria had been maintained in HeLa, Caco-2, LS174T, and THP-1 cells for 18 h, as indicated by asterisks (*p < 0.05; n = 3).

Putrescine, Spermine, and Cadaverine Are the Major Polyamines in S. Typhimurium, Regardless of speG and Internalization in Caco-2 Cells

To quantify the concentrations of putrescine, cadaverine, spermidine, and spermine in S. Typhimurium SL1344 before and after invasion in Caco-2 cells and to investigate whether speG affects their concentrations in S. Typhimurium SL1344 outside and inside Caco-2 cells, as well as extracellular and intracellular S. Typhimurium SL1344, we performed HPLC to quantify these polyamines from the mid-log cultures of both strains and from their 18-h postinfection intracellular concentrations in Caco-2 cells. Our HPLC analysis of the mid-log cultures
of S. Typhimurium SL1344 revealed that the concentration of putrescine (47.5 mM), the predominant polyamine, was approximately six times higher than that of spermine (8.0 mM) and approximately 10 times higher than that of cadaverine (4.8 mM). The concentration of spermidine (0.2 mM) was the lowest (Figure 2A). The order of the cellular contents of all
these cytoplasmic polyamines was the same in *S. Typhimurium* after the deletion of *speG* (Figure 2B), as well as in intracellular *S. Typhimurium* and Δ*speG* (Figures 2C,D). In summary, putrescine, spermine, and cadaverine were the polyamines with the highest concentrations in extracellular and intracellular *S. Typhimurium* SL1344 and Δ*speG* (Figures 2A–D).

**speG Suppresses the Concentration of Spermine but Increases That of Cadaverine in Extracellular *S. Typhimurium***

To investigate the effects of *speG* on the contents of polyamines in *S. Typhimurium*, the HPLC-quantified concentrations of the polyamines in *S. Typhimurium* SL1344 and Δ*speG* were compared. *S. Typhimurium ΔspeG* contained significantly lower concentrations of cadaverine and higher concentrations of spermine than did *S. Typhimurium* SL1344 (4.1 vs. 4.8 mM and 16.5 vs. 8.0 mM, respectively; Figures 2F,H), indicating that *speG* expression might enhance cadaverine production and suppress spermine synthesis. The deletion of *speG* did not significantly change the content of spermidine in *S. Typhimurium*.

**Biosynthesis of Putrescine, Cadaverine, and Spermidine Is Reduced in Intracellular *S. Typhimurium* in Caco-2 Cells and *speG* Is Involved in Inhibition of Polyamine Production in Intracellular *S. Typhimurium***

After bacterial invasion in Caco-2 cells for 18 h, the concentrations of putrescine, cadaverine, and spermidine significantly decreased in *S. Typhimurium* SL1344 (32.8 vs. 47.5 mM, 1.8 vs. 4.8 mM, and 0.2 vs. 0.1 mM, respectively; Figures 2E–G); however, the levels of spermine were similar to those in extracellular *S. Typhimurium* SL1344 (7.6 vs. 8.8 mM; Figure 2H). Briefly, the biosynthesis of putrescine, cadaverine, and spermidine was significantly suppressed in *S. Typhimurium* SL1344 in Caco-2 cells 18 h after invasion. To verify the hypothesis that deficiency in the intracellular replication of *S. Typhimurium ΔspeG* is due to its quantitative alteration in polyamines, the concentrations of four polyamines were compared between intracellular *S. Typhimurium* SL1344 and Δ*speG*. We observed that the concentrations of putrescine, spermine, and cadaverine were higher in intracellular *S. Typhimurium ΔspeG* than in intracellular *S. Typhimurium* SL1344 (Figures 2C,D). Briefly, the deletion of *speG* significantly increased the concentrations of cadaverine, spermidine, and spermine in intracellular *S. Typhimurium* in Caco-2 cells for 18 h (3.7 vs. 1.8 mM, 0.2 vs. 0.1 mM, and 13.7 vs. 7.6 mM, respectively; Figures 2F–H) and non-significantly increased the concentration of putrescine (43.2 vs. 32.8 mM, *p* = 0.093; Figures 2E). These results suggested that *speG* can inhibit the biosynthesis of polyamines in intracellular *S. Typhimurium*, with a different tendency for modulating putrescine, cadaverine, and spermidine compared with extracellular *S. Typhimurium*.

**Intracellular Proliferation of *S. Typhimurium* Is Dose-Dependently Suppressed by Putrescine, Cadaverine, and Spermine***

Because non-replicating intracellular *S. Typhimurium ΔspeG* contains higher concentrations of putrescine, spermine, and cadaverine, we hypothesized that the accumulation of these polyamines inhibits the intracellular replication of *S. Typhimurium* SL1344. A polyamine suppression assay similar to the 18-h intracellular replication assay was performed by infecting Caco-2 cells with *S. Typhimurium* SL1344 for 2 h, followed by 1-h treatment with gentamicin to kill extracellular bacteria and 15-h coincubation of Caco-2 cells with intracellular *S. Typhimurium* SL1344 and the three polyamines in two estimated concentrations. The HPLC analysis of polyamines in intracellular *S. Typhimurium ΔspeG* was conducted after 2-h amplification through the shaking incubation of bacteria released from lysed Caco-2 cells, and the doubling time of *S. Typhimurium* was 20–30 min. Therefore, the estimated concentrations of putrescine (625 and 312 µM), cadaverine (125 and 62.5 µM), and spermine (375 and 187.5 µM) were 25 to 26 times diluted compared with their corresponding concentrations (43.2, 3.7, and 13.7 mM) in the HPLC quantification (intracellular *S. Typhimurium ΔspeG*; Figure 2D). Intracellular *S. Typhimurium* SL1344 (1.1 × 107 CFU per inoculum of 107 CFU) was significantly suppressed by 625 and 312.5 µM of putrescine (7.2 × 107 CFU per inoculum of 107 CFU, *p* = 0.006, and 8.5 × 107 CFU per inoculum of 107 CFU, *p* = 0.04, respectively), 125 µM of cadaverine (7.7 × 107 CFU per inoculum of 107 CFU, *p* = 0.017), and 375 and 187.5 µM of spermine (7.1 × 107 CFU per inoculum of 107 CFU, *p* = 0.015 and 9 × 107 CFU per inoculum of 107 CFU, *p* = 0.04, respectively). The concentration of intracellular *S. Typhimurium* SL1344 was non-significantly lower in Caco-2 cells treated with 625.5 µM of cadaverine (9.2 × 107 CFU per inoculum of 107 CFU, *p* = 0.138) than that of Caco-2 cells not treated with polyamines (Figure 3). Altogether, *S. Typhimurium* SL1344 was dose-dependently suppressed by putrescine, cadaverine, and spermine (Figure 3).

**speG Is Involved in Suppressing Upregulation of Genes Associated with Periplasmic Nitrate Reductase System, Glucarate Metabolism, Phosphotransferase System, Cytochromes, and Succinate Reductase Complex***

To investigate the effects of *speG* on the expression of other genes in *S. Typhimurium*, the entire RNA transcriptome of *S. Typhimurium ΔspeG* was compared with that of *S. Typhimurium* SL1344 (*Data Sheet 1*). In the mid-log cultures, 29 genes were significantly upregulated in *S. Typhimurium ΔspeG* compared with *S. Typhimurium* SL1344, including *yojF* and *napF*, which are involved in the periplasmic nitrate reductase system; *ygcX, ygcZ, garL, garR*, and SL1344_2942,
which are associated with glucarate metabolism; SL1344_3736 and SL1344_4467, which are related to the phosphotransferase system; and SL1344_4467, which are involved in suppression of the expression of these significantly upregulated genes in the succinate reductase complex (Table 2A). Moreover, the depletion of speG led to the significant upregulation of 11 genes in intracellular S. Typhimurium, namely ilvC, ilvG, ilvA, leuD, leuC, and leuB in the ilv-leu operon and hisG and hisC in the histidine operon (Table 3A). Only four genes, namely speG, smvA, pyre, and citD2, were significantly downregulated in the intracellular S. Typhimurium ΔspeG mutant (Table 3B). The RNA microarray results were validated through qRT-PCR, which revealed the significantly upregulated mRNA expression of four genes (ilvC, leuD, hisG, and SL1344_2430) and significantly downregulated mRNA expression of three genes (smvA, pyre, and speG; Figure 4B).

**speG Is Involved in Suppressing Upregulation of Genes in the ilv-leu Operon and Histidine Operon of Intracellular S. Typhimurium after Invasion in Caco-2 Cells**

To understand whether speG affects the gene expression of intracellular S. Typhimurium, the transcriptomes of intracellular S. Typhimurium SL1344 and its ΔspeG mutant were compared after internalization in Caco-2 cells for 18 h. Compared with the expression pattern in intracellular S. Typhimurium SL1344, 1,964 genes were upregulated and 2,664 genes were downregulated in the intracellular S. Typhimurium ΔspeG mutant (Data Sheet 2). The depletion of speG led to the significant upregulation of six flagellar genes (flhA, flhB, flfP, flfQ, flgI, and flhI) and four fimbrial genes (fimC, fimD, fimL, and fimW; Figure 4A).

**speG Affects Flagellation and Motility of S. Typhimurium and Exogenous Polyamins Attenuate Motility of S. Typhimurium**

To validate the RNA microarray data on the involvement of speG in flagellar biosynthesis, the bacterial morphology and motility of S. Typhimurium SL1344 and ΔspeG were examined. Transmission electron micrographs after negative staining revealed sparse defective flagella in S. Typhimurium ΔspeG compared with the wild-type SL1344 strain (Figure 5). Moreover, the motility of S. Typhimurium ΔspeG was poorer than that of the wild-type SL1344 strain but better than that of S. Typhimurium ΔflhC (Figure 6). The bacterial motility was restored after speG complementation in the ΔspeG mutant (Figure 6C), suggesting the influence of speG on bacterial motility. In addition, the motility of S. Typhimurium SL1344 was dose-dependently attenuated by exogenous putrescine, cadaverine, and spermidine (Figure 7).
TABLE 2 | The 29 significantly upregulated genes (A) and the 110 significantly downregulated genes (B) of the S. Typhimurium ΔspeG mutant compared with its parental wild-type strain SL1344.

| Gene name | Target ID | Function | Log2 fold change |
|-----------|-----------|----------|-----------------|
| (A) UPREGULATED GENES | | | |
| Genes of Periplasmic Nitrate Reductase System | | | |
| yojF | SL1344_2230 | Putative napAB assembly protein | 3.782 |
| napF | SL1344_2231 | Ferredoxin-type protein NapF | 3.073 |
| Genes of Glucarate Metabolism | | | |
| ygcZ | SL1344_2943 | Glucarate transporter | 3.248 |
| garL | SL1344_3222 | 5-Keto-4-deoxy-D-glucarate aldolase | 2.819 |
| garR | SL1344_3221 | 2-Hydroxy-3-oxopropionate reductase | 2.760 |
| ygcX | SL1344_2941 | Glucarate dehydratase | 2.037 |
| SL1344_2942 | SL1344_2942 | Glucarate dehydratase | 1.257 |
| Genes of Phosphotransferase System | | | |
| SL1344_3736 | SL1344_3736 | Putative PTS system protein | 1.673 |
| SL1344_4467 | SL1344_4467 | PTS transport system, IIB component | 1.621 |
| Genes of Cytochromes | | | |
| cyaA | SL1344_0437 | Cytochrome o ubiquinol oxidase subunit II | 1.668 |
| cyaB | SL1344_0436 | Cytochrome o ubiquinol oxidase subunit I | 1.406 |
| cyaC | SL1344_0716 | Cytochrome o ubiquinol oxidase subunit III | 1.227 |
| Genes of Succinate Dehydrogenase Complex | | | |
| scdH | SL1344_0715 | Succinate dehydrogenase hydrophobic membrane anchor protein | 1.239 |
| scdA | SL1344_0435 | Succinate dehydrogenase flavoprotein subunit | 1.121 |
| scdB | SL1344_0717 | Succinate dehydrogenase iron-sulfur protein | 1.120 |
| scdC | SL1344_0714 | Succinate dehydrogenase cytochrome b-556 subunit | 1.027 |
| Other Genes | | | |
| narK | SL1344_1693 | Nitrite extrusion protein | 4.443 |
| malK | SL1344_4167 | Maltose-maltodextrin transport ATP-binding protein | 3.343 |
| ybfM | SL1344_0669 | Putative outer membrane protein | 2.372 |
| SL1344_2997 | SL1344_2997 | Acetyl-CoA acetyltransferase | 2.089 |
| SL1344_3662 | SL1344_3662 | Putative racemase | 1.552 |
| SL1344_0211 | SL1344_0211 | Hypothetical protein | 1.496 |
| dctA | SL1344_3579 | G4-dicarboxylate transport protein | 1.454 |
| SL1344_2940 | SL1344_2940 | Hypothetical protein | 1.319 |
| hutU | SL1344_0767 | Urocanate hydratase | 1.237 |
| SL1344_3732 | SL1344_3732 | Hypothetical protein | 1.227 |
| SL1344_0790 | SL1344_0790 | Hypothetical protein | 1.163 |
| SL1344_1227 | SL1344_1227 | Hypothetical protein | 1.010 |
| hutH | SL1344_0768 | Histidine ammonia-lyase | 1.002 |
| (B) DOWNREGULATED GENES | | | |
| Flagellar Genes | | | |
| fliA | SL1344_1848 | Flagellar biosynthetic protein FlhA | −1.994 |
| fliB | SL1344_1849 | Flagellar biosynthetic protein FlhB | −1.918 |
| fliP | SL1344_1908 | Flagellar biosynthetic protein FlIP | −1.776 |
| fliB | SL1344_1887 | Flagellin lysine-N-methylase | −1.751 |
| fliQ | SL1344_1909 | Flagellar biosynthetic protein FlIQ | −1.688 |
| fgi | SL1344_1118 | Flagellar P-ring protein | −1.648 |
| fliN | SL1344_1906 | Flagellar motor switch protein FlIN | −1.645 |
| fliO | SL1344_1907 | Flagellar biosynthesis protein | −1.639 |
| fliF | SL1344_1898 | Flagellar basal-body M-ring protein | −1.633 |
| fliE | SL1344_1847 | Flagellar protein FHe | −1.603 |
| fliR | SL1344_1910 | Flagellar biosynthetic protein FlIR | −1.601 |

(Continued)
**TABLE 2 | Continued**

| Gene name     | Target ID       | Function                                                                 | Log2 fold change |
|---------------|-----------------|--------------------------------------------------------------------------|------------------|
| flgH          | SL1344_1117     | Flagellar L-ring protein                                                  | −1.586           |
| flgL          | SL1344_1121     | Flagellar hook-associated protein 3                                      | −1.516           |
| flgJ          | SL1344_1119     | Flagellar protein FlgJ                                                   | −1.507           |
| flmM          | SL1344_1905     | Flagellar motor switch protein FlmM                                      | −1.473           |
| flmA          | SL1344_1885     | RNA polymerase sigma transcription factor for flagellar operon            | −1.458           |
| flgL          | SL1344_1904     | Flagellar biosynthesis protein                                            | −1.415           |
| flgJ          | SL1344_1902     | Flagellar biosynthesis protein                                            | −1.401           |
| flgK          | SL1344_1903     | Flagellar hook-length control protein                                     | −1.361           |
| flgI          | SL1344_1900     | Flagellar assembly protein FlgI                                           | −1.339           |
| flgG          | SL1344_1899     | Flagellar motor switch protein FlgG                                       | −1.316           |
| flgD          | SL1344_1889S    | Flagellar hook associated protein FlgD                                   | −1.251           |
| flgS          | L1344_1890      | Flagellar protein FlgS                                                   | −1.211           |
| flgT          | SL1344_1891     | Flagellar protein FlgT                                                   | −1.056           |
| **Fimbrial Genes** |               |                                                                          |                  |
| fimW          | SL1344_0545     | Fimbriae w protein                                                       | −2.084           |
| fimC          | SL1344_0538     | Fimbrial chaperone protein                                                | −1.694           |
| fimD          | SL1344_0539     | Outer membrane usher protein FimD                                        | −1.623           |
| fimI          | SL1344_0537     | Major pilin protein                                                       | −1.442           |
| stfC          | SL1344_4500     | Fimbrial chaperone                                                        | −1.386           |
| **T3SS-Related Genes** |           |                                                                          |                  |
| sopD          | SL1344_2924     | Hypothetical protein                                                      | −3.273           |
| sopA          | SL1344_2043     | Secreted protein SopA                                                     | −3.273           |
| sopE2         | SL1344_1784     | Invasion-associated secreted effector protein (sopE2)                     | −2.996           |
| slfP          | SL1344_0776     | Leucine rich repeat                                                       | −2.537           |
| spiR          | SL1344_1326     | Putative two-component sensor kinase                                       | −1.974           |
| ssrB          | SL1344_1325     | Putative two-component response regulator                                  | −1.705           |
| srfC          | SL1344_1526     | Putative virulence effector protein                                       | −1.506           |
| srfA          | SL1344_1524     | Putative virulence effector protein                                       | −1.451           |
| srfB          | SL1344_1525     | Putative virulence effector protein                                       | −1.361           |
| ssal          | SL1344_1342     | Putative pathogenicity island protein                                     | −1.324           |
| ssaj          | SL1344_1343     | Putative pathogenicity island lipoprotein                                 | −1.230           |
| ssaA          | SL1344_1331     | T3SS chaperone                                                            | −1.222           |
| ssaH          | SL1344_1341     | Type three secretion system apparatus                                      | −1.218           |
| sseB          | SL1344_1332     | Putative pathogenicity island effector protein                            | −1.129           |
| ssaG          | SL1344_1340     | Putative pathogenicity island protein                                     | −1.019           |
| **Other Genes** |                   |                                                                          |                  |
| speG          | SL1344_1432     | Spermidine N1-acetyltransferase                                           | −8.818           |
| asnA          | SL1344_3844     | Asparagine synthetase A                                                   | −3.380           |
| SL1344_3163   | SL1344_3163     | Hypothetical protein                                                      | −3.327           |
| pipB          | SL1344_1027     | Hypothetical protein                                                      | −3.148           |
| slE           | SL1344_4197     | Hypothetical protein                                                      | −3.113           |
| pipC          | SL1344_1029     | Cell invasion protein                                                     | −2.944           |
| SL1344_1265   | SL1344_1265     | Putative DNA/RNA non-specific endonuclease                                | −2.843           |
| siaF          | SL1344_4198     | Putative type-1 secretion protein                                         | −2.823           |
| SL1344_0337   | SL1344_0337     | Hypothetical protein                                                      | −2.473           |
| SL1344_1028   | SL1344_1028     | Inner membrane protein                                                    | −2.278           |
| ugtL          | SL1344_1531     | Hypothetical protein                                                      | −2.168           |
| SL1344_1846   | SL1344_1846     | Hypothetical protein                                                      | −2.072           |
| pagD          | SL1344_1183     | Putative outer membrane virulence protein (PagD)                          | −2.006           |
| SL1344_1330A  | SL1344_1330A    | Hypothetical protein                                                      | −1.957           |

(Continued)
| Gene name | Target ID | Function | Log2 fold change |
|-----------|-----------|----------|-----------------|
| SL1344_1796 | SL1344_1796 | Hypothetical protein | −1.932 |
| SL1344_4434 | SL1344_4434 | Hypothetical protein | −1.929 |
| baeS | SL1344_2107 | Putative two-component system sensor kinase | −1.907 |
| pagC | SL1344_1184 | Outer membrane invasion protein (PagC) | −1.863 |
| SL1344_2763 | SL1344_2763 | Hypothetical protein | −1.827 |
| SL1344_1083 | SL1344_1083 | Hypothetical protein | −1.765 |
| SL1344_4435 | SL1344_4435 | Hypothetical protein | −1.721 |
| SL1344_1794 | SL1344_1794 | Putative inner membrane protein | −1.705 |
| SL1344_1530A | SL1344_1530A | Hypothetical protein | −1.680 |
| SL1344_1783 | SL1344_1783 | Hypothetical protein | −1.666 |
| SL1344_1867 | SL1344_1867 | Putative lipoprotein | −1.654 |
| gtgE | SL1344_0995 | Bacteriophage encoded virulence factor | −1.626 |
| SL1344_0532 | SL1344_0532 | Hypothetical protein | −1.615 |
| pagO | SL1344_1793 | Inner membrane protein (PagO) | −1.606 |
| lap | SL1344_2915 | Alkaline phosphatase isozyme conversion protein | −1.604 |
| SL1344_0544 | SL1344_0544 | Hypothetical protein | −1.597 |
| SL1344_0973 | SL1344_0973 | Bacteriophage protein | −1.467 |
| yegB | SL1344_2106 | Putative transporter protein | −1.428 |
| SL1344_2984 | SL1344_2984 | Hypothetical protein | −1.425 |
| SL1344_0947 | SL1344_0947 | Bacteriophage protein | −1.421 |
| SL1344_0974 | SL1344_0974 | Bacteriophage protein | −1.415 |
| SL1344_1345 | SL1344_1345 | Putative pathogenicity island protein | −1.389 |
| hyaD2 | SL1344_1465 | Hydrogenase 1 maturation protease | −1.376 |
| SL1344_0948 | SL1344_0948 | Bacteriophage protein | −1.361 |
| hyaF2 | SL1344_1462 | Hydrogenase-1 operon protein HyaF2 | −1.341 |
| SL1344_0531 | SL1344_0531 | Putative exported outer membrane protein | −1.337 |
| SL1344_1085 | SL1344_1085 | Hypothetical protein | −1.336 |
| cheA | SL1344_1856 | Chemotaxis protein CheA | −1.317 |
| asnB | SL1344_0662 | Asparagine synthetase B | −1.314 |
| SL1344_0739 | SL1344_0739 | Putative hydrolyase | −1.305 |
| SL1344_1188 | SL1344_1188 | Hypothetical protein | −1.286 |
| SL1344_0975 | SL1344_0975 | Bacteriophage protein | −1.269 |
| phoN | SL1344_4255 | Non-specific acid phosphatase | −1.248 |
| C1 | SL1344_0961 | Transcriptional activator-regulatory protein | −1.238 |
| ybcI | SL1344_0533 | Hypothetical protein | −1.210 |
| econR | SL1344_4274 | Transcriptional regulator | −1.204 |
| SL1344_1928 | SL1344_1928 | Putative bacteriophage protein | −1.193 |
| gpO | SL1344_2591 | Bacteriophage replication protein | −1.136 |
| SL1344_3128 | SL1344_3128 | Hypothetical protein | −1.111 |
| SL1344_0993 | SL1344_0993 | Bacteriophage protein | −1.107 |
| SL1344_1464 | SL1344_1464 | Hydrogenase isoenzymes formation protein | −1.103 |
| yfeA | SL1344_2378 | Hypothetical protein | −1.103 |
| pphA | SL1344_1782 | Serine–threonine protein phosphatase | −1.101 |
| yicC | SL1344_4200 | Hypothetical protein | −1.074 |
| SL1344_1628 | SL1344_1628 | Hypothetical protein | −1.049 |
| SL1344_3130 | SL1344_3130 | Hypothetical protein | −1.034 |
| SL1344_1461 | SL1344_1461 | Putative ATP/GTP-binding protein | −1.029 |
| pglE | SL1344_2363 | Outer membrane protease E | −1.024 |
| SL1344_1344 | SL1344_1344 | Putative pathogenicity island protein | −1.013 |
| hyaE2 | SL1344_1463 | Hydrogenase-1 operon protein HyaE2 | −1.006 |
| rtcB | SL1344_3486 | Hypothetical protein | −1.005 |
| SL1344_1845 | SL1344_1845 | Penicillin-binding protein | −1.002 |
DISCUSSION

According to our review of relevant literature, the present study is the first to demonstrate that speG is required for the intracellular replication of Salmonella in human non-phagocytic cells. Our results revealed that the effect of speG on the intracellular replication of S. Typhimurium in HeLa cells is time dependent (Figure 1A). Moreover, we observed that speG influenced the intracellular replication of S. Typhimurium in four cell lines as hosts (Figures 1B–E). Although the speG mutant was attenuated in bacterial invasion in Caco-2 cells, it was more significantly attenuated in bacterial replication after 18-h internalization in Caco-2 cells (Figure 1C), suggesting the effect of speG on the suppression of intracellular bacterial replication. The depletion of speG did not affect the internalization of S. Typhimurium in THP-1 cells (Figure 1E), which indicated that the speG phenotype is mainly exhibited within host cells rather than outside the host cells. In E. coli, speG encodes SAT, which catalyzes spermidine to N1- or N8-acethylspermidine (Igarashi and Kashiwagi, 2010; Barbagallo et al., 2011; Jelsbak et al., 2012), which can be secreted by the bacteria to inactivate the toxicity of excessive spermidine (Fukuchi et al., 1995) and can reduce intracellular polyamine levels to prevent spermidine toxicity under stressful growth conditions (Limsuwun and Jones, 2000). The amino acid sequence of SAT in E. coli is similar to that in Enterococcus faecalis, Pseudomonas aeruginosa plasmid pSCH884, Klebsiella pneumoniae, Serratia marcescens, and Agrobacterium tumefaciens (Fukuchi et al., 1994). However, speG is not present in all bacteria and is lost in Shigella, resulting in higher concentrations of endogenous spermidine (Barbagallo et al., 2011). Although the absence of speG enhances the survival of Shigella to oxidative stress and the adverse environments inside
### Table 3

The 11 significantly upregulated genes (A) and the four significantly downregulated genes (B) of the intracellular *S. Typhimurium* ΔspeG mutant compared with its parental wild-type strain SL1344 within Caco-2 cells.

| Gene Target ID | Function | Log2 fold change |
|----------------|----------|-----------------|
| **(A) SIGNIFICANTLY UPREGULATED GENES** | | |
| ilvC SL1344_3869 | Ketol-acid reductoisomerase | 1.733 |
| leuD SL1344_0110 | 3-Isopropylmalate dehydratase small subunit | 1.694 |
| leuC SL1344_0111 | 3-Isopropylmalate dehydratase large subunit | 1.670 |
| hisG SL1344_2048 | ATP phosphoribosyltransferase | 1.499 |
| ilvM SL1344_3862 | Acetoxyhydrox acid synthase II, small subunit | 1.351 |
| leuB SL1344_0112 | 3-Isopropylmalate dehydrogenase | 1.333 |
| ilvG SL1344_3861 | Acetolactate synthase large subunit | 1.141 |
| ilvA SL1344_3865 | Threonine deaminase | 1.077 |
| SL1344_2430 | Putative cobalamin adenosyltransferase | 1.052 |
| SL1344_1416 | Putative membrane transport protein | 1.045 |
| hisC SL1344_2050 | Histidinol-phosphate aminotransferase (imidazole) | 1.029 |
| **(B) SIGNIFICANTLY DOWNREGULATED GENES** | | |
| speG SL1344_1432 | Spermidine N1-acetyltransferase | -6.578 |
| smvA SL1344_1505 | Methyl viologen resistance protein SmvA | -2.885 |
| pyrE SL1344_3699 | Orotate phosphoribosyltransferase | -1.207 |
| citD2 SL1344_0060 | Citrate lyase acyl carrier protein | -1.043 |

### Figure 5

Transmission electron micrographs of *S. Typhimurium* SL1344 and ΔspeG. Transmission electron micrographs after negative staining revealed the morphology of (A,B) *S. Typhimurium* SL1344 and (C,D) ΔspeG. Numerous long flagella were observed in *S. Typhimurium* SL1344 [magnification: (A) 10,000× and (B) 22,500×]. Only a small number of fragmented flagella were observed in *S. Typhimurium* ΔspeG [magnification: (C) 10,000× and (D) 22,500×].
Barbagallo et al., 2011). Moreover, we observed...casualino). These observations are consistent
with our results that the loss of speG in S. Typhimurium did
not impair bacterial invasion in HeLa cells but attenuated its
intracellular replication (Figure 1). Such a phenotype of speG
associated with the intracellular replication of S. Typhimurium
is significantly expressed after bacterial invasion in cells for a
sufficiently long duration (Figures 1B–E) rather than for short
durations (Figure 1A), possibly because of the time-dependent
accumulation of intracellular metabolites caused by impaired
SAT synthesis in the speG mutant. Therefore, whether the
accumulation of spermidine in Salmonella is detrimental to
to bacterial intracellular proliferation expedited our subsequent
quantification of the four major polyamines in S. Typhimurium
and its speG-deleted mutant outside and inside Caco-2 cells.

This study was the first to report that putrescine, spermine, and cadaverine are the polyamines with the highest concentrations and that the concentration of spermidine is the lowest among the four major polyamines in S. Typhimurium, regardless of whether speG is in the bacterial genome. Our HPLC analysis indicated that putrescine is the predominant polyamine in S. Typhimurium SL1344, followed by spermine and cadaverine. The same order was observed in extracellular S. Typhimurium ΔspeG, as well as in intracellular S. Typhimurium SL1344 and ΔspeG in Caco-2 cells (Figure 2). Consistent with our result, a recent study used gas chromatography mass spectrometry and reported that putrescine has the highest positive loading in intracellular profiles of S. Typhimurium, just second to succinic acid (Wong et al., 2015). Similar to E. coli, putrescine is the most abundant polyamine in S. Typhimurium. It is the main constituent of the outer membrane of both bacteria (Koski and Vaara, 1991; Cohen, 1997; Shah and Swiatlo, 2008). Moreover, we observed that S. Typhimurium contains a high concentration of spermine and cadaverine, both of which are scarce or absent in E. coli (Cohen, 1997; Shah and Swiatlo, 2008). This study was the first to address the relevance of spermine and cadaverine in invasive bacteria such as Salmonella. Shigella and all enteroinvasive E. coli strains lack lysine decarboxylase for the decarboxylation of lysine to synthesize cadaverine, a small polyamine that inhibits the inflammation induced by dysentery bacteria (Casalino et al., 2003). Therefore, the high cellular levels of cadaverine suppressing host inflammation could explain the resistance of Salmonella to the intracellular immunity of host cells. Piperidine, a cadaverine metabolite, dose-dependently inhibits the virulence of S. Typhimurium during pathogen-host interactions in vitro and in vivo (Kohler et al., 2002). Further studies must determine how the orchestration of endogenous and exogenous cadaverine modulates the pathogenesis of Salmonella. In contrast to the loss of speG in Shigella inducing the accumulation of intracellular spermidine, which favors bacterial survival under oxidative stress conditions (Campilongo et al., 2014), the intracellular proliferation and survival of S. Typhimurium is independent of the accumulation of intracellular spermidine because it has considerably lower concentration of spermidine than of the polyamines in bacterial cells (Figure 2). Furthermore, numerous cellular transport systems can regulate the levels of

![Figure 6](image_url)
polyamine in bacteria. *E. coli* exerts polyamine import by the spermidine-preferential PotABCD, putrescine-specific PotFGHI and PuuP transporters (Kashiwagi et al., 1993; Igarashi et al., 2001; Kurihara et al., 2009), putrescine–ornithine exchange by PotE (Kashiwagi et al., 1997), cadaverine–lysine exchange by CadB (Soksawatmaekhin et al., 2004), spermidine excretion by MdtJI (Higashi et al., 2008), and putrescine export by SapBCDF (Sugiyama et al., 2016). Nevertheless, speG did not significantly affect the expression of these genes in *S. Typhimurium* in our study.

**speG**-regulated polyamine synthesis in *S. Typhimurium* varies before and after invasion in human intestinal epithelial cells. Our HPLC analysis indicated that the depletion of *speG* significantly and constantly increased the concentrations of spermine before and after invasion in Caco-2 cells (Figures 2B,D), suggesting that *speG* affects the inhibition of spermine synthesis without being influenced by the intracellular environments of host cells. Contrastingly, the effect of *speG* on intracellular *S. Typhimurium* after 18-h internalization in Caco-2 cells exhibited a different phenotype by modulating the cytoplasmic contents of putrescine, cadaverine, and spermidine, namely the intracellular inhibition of the accumulation of these polyamines. The knockout effect of *speG* on the suppression of the contents of putrescine and cadaverine in extracellular *S. Typhimurium* was the reverse of the enhancement of these polyamines after the invasion of *S. Typhimurium* in Caco-2 cells. *speG* did not influence the concentration of spermidine in extracellular *S. Typhimurium* but significantly increased it in intracellular *S. Typhimurium*.

**FIGURE 7** | Polyamine-suppressing bacterial motility assays of *S. Typhimurium* SL1344. The motilities of *S. Typhimurium* SL1344 were examined by bacterial inoculation on semisolid agar plates supplemented with four polyamines after 6-h incubation at 37°C. The motility zones of *S. Typhimurium* SL1344 in the semisolid LB agar plates supplemented with putrescine (625 µM; G), cadaverine (125 µM; E), and spermidine (6 µM; I) were smaller than that of *S. Typhimurium* SL1344 in the plates containing no polyamine (A). The motility zones of *S. Typhimurium* SL1344 were slightly inhibited by cadaverine (62.5 µM; D), but not suppressed by putrescine (312.5 µM; B), spermidine (3 µM; F), and two concentrations of spermine (187.5 µM; H and 375 µM; I) in the semisolid LB agar plates.
Until now, most studies have investigated polyamines in *E. coli*. In *E. coli*, putrescine is synthesized as in mammalian cells via two pathways, either by the catalysis of L-ornithine by ornithine decarboxylase (SpeC/SpecF), as in mammalian cells, or by the catalysis of L-arginine by arginine decarboxylase (SpeA) into agmatine, which is further catalyzed by agmatine ureohydrolase (SpeB). However, spermidine is synthesized from putrescine and decarboxylated S-adenosylmethionine catalyzed by spermidine synthase (SpeE), with the simultaneous metabolism of decarboxylated S-adenosylmethionine to methylthioadenosine. S-adenosylmethionine decarboxylase (SpeD) catalyzes the synthesis of decarboxylated S-adenosylmethionine from S-adenosylmethionine that is originally metabolized from its upstream precursor L-methionine catalyzed by MetK with ATP consumption. Theoretically, the impaired function of SpeG leads directly to the accumulation of spermidine or indirectly to its precursor metabolites, namely putrescine, agmatine, arginine, or ornithine. Unlike the loss of *speG* in *Shigella* that results in higher concentrations of endogenous spermidine (Barbagallo et al., 2011), our study indicated that the genuine *speG* effect is mainly dependent on spermine, rather than on spermidine, in both extracellular and intracellular *S. Typhimurium*, with its versatile modulation on putrescine and cadaverine affected by the intracellular environment of host cells. Considering our finding regarding the effect of *speG* on the intracellular replication of *S. Typhimurium*, we hypothesized that the loss of *speG* could result in the marked accumulation of putrescine, spermine, and cadaverine in intracellular *S. Typhimurium* or affect the expression of other *Salmonella* virulence genes via an unknown pathway to hinder intracellular bacterial replication.

The accumulation of cellular polyamines, including putrescine, spermine, and cadaverine, impedes the intracellular replication of *Salmonella* in human intestinal epithelial cells. Polyamines are indispensable for normal cell growth and affect the stimulation of cell division and proliferation; gene expression for the survival of cells, the regulation of apoptosis, oxidative stress, cell-cell communication, and the synthesis and functions of DNA and protein synthesis, particularly RNA because most of cellular polyamines exist in a polyamine–RNA complex in cells (Igarashi and Kashiwagi, 2010; Lenis et al., 2017). However, studies have rarely determined whether insufficient or excessive polyamines are detrimental to bacterial proliferation. Decreased concentrations of spermidine and putrescine reduce the growth rate of *E. coli* (Cunningham-Rundles and Maas, 1975; Xie et al., 1993). A study reported that exogenous spermine dose-dependently inhibits the *in vitro* growth of *S. Typhimurium*, *E. coli*, and *Staphylococcus aureus*, but not *P. aeruginosa* (Kwon and Lu, 2007). To validate our observation of the increased polyamine contents of non-replicating intracellular *S. Typhimurium ΔspeG*, we demonstrated that the intracellular proliferation of *S. Typhimurium* was dose-dependently suppressed by putrescine, cadaverine, and spermidine in the concentrations estimated from our earlier HPLC analysis (Figure 3). Recent studies have reported several cellular mechanisms controlling the levels of intracellular polyamines fine-tuned by their biosynthesis, catabolism, and transport at the transcription, translation, and protein degradation levels involving feedback loops controlled by polyamine concentrations. These mechanisms include the transcriptional and translational control of ornithine decarboxylase, regulation of polyamine synthesis by antizyme levels, catalysis of S-adenosylmethionine into decarboxylated S-adenosylmethionine for synthesizing spermidine and spermine from putrescine, controlling polyamine levels through catabolism by oxidation or acetylation to maintain their cellular activity or concentration, and controlling polyamine levels through transport (Filippou et al., 2007; Igarashi and Kashiwagi, 2010; Miller-Fleming et al., 2015; Lenis et al., 2017). Furthermore, the function of polyamines in *S. Typhimurium* had remained largely unknown until the transcriptome of intracellular *S. Typhimurium* revealed the upregulation of genes for putrescine and spermidine biosyntheses during the infection of epithelial cells and macrophages, suggesting an important role of polyamines in bacterial invasion and intracellular survival (Eriksson et al., 2003; Haufert et al., 2008; Di Martino et al., 2013). A polyamine mutant of *S. Typhimurium* with *speB, speC, speE*, and *speF* deletions exhibited a defective invasion of epithelial cells and attenuation in intracellular replication compared with its wild-type counterpart and the typhoid mouse model (Jelsbak et al., 2012). This defective intracellular replication was enhanced by the complementation of *speB* in the polyamine mutant and exogenous putrescine and spermidine in the culture media before the infection of the cell cultures (Jelsbak et al., 2012). These results indicated a critical role of putrescine and spermidine in controlling virulence in *S. Typhimurium*, most likely through the upregulation of essential virulence loci in SPI-1 and SPI-2 (Jelsbak et al., 2012). However, the genotypes of *speG* in bacterial virulence and their role in regulating the toxicity of excessive polyamines in *Salmonella* remain unclear.

The relationship of polyamines with bacterial flagellation and motility in *Salmonella* has rarely been reported. Polyamines may be involved in the intracellular virulence of *S. Typhimurium*. An early study reported that a housekeeping gene, *sifA*, located within the *potABCD* operon and involved in the periplasmic transport of polyamines, is required for synthesizing *Salmonella*-induced filaments in epithelial cells and for *in vivo* virulence in mice (Stein et al., 1996). Our study demonstrated that *speG* contributes not only to intracellular replication in host cells but also the flagellar biosynthesis and swimming motility of *S. Typhimurium*, suggesting that the *speG*-associated metabolism of polyamines accounts for bacterial morphology and motility and is associated with intracellular growth. In addition, we demonstrated remarkable dose-dependent suppression of *Salmonella* motility by exogenous putrescine, cadaverine, and spermidine. However, *speG* did not significantly affect the expression of other polyamine genes, suggesting that *speG* affects the flagellation of *Salmonella* independently of *speA*, *speB*, *speC*, *speD*, *speE*, *cadA*, and *metK*. The production of flagellar protein requires the concomitant synthesis of RNA in *S. Typhimurium* (Aamodt and Eisenstadt, 1968). Because polyamines in a polyamine–RNA complex in cells are essential for RNA synthesis and functions (Igarashi and Kashiwagi, 2010; Lenis et al., 2017), it is reasonably assumed that the unbalanced metabolism of polyamines could interfere with
the flagellation of *S. Typhimurium*. A recent proteomic study confirmed the suppression of bacterial flagellation, chemotaxis, SPI-1, TCA cycle, and anaerobic respiration pathways in the metabolic reshuffling of intracellular-replicating *Salmonella* in infected HeLa cells (Liu et al., 2017). Furthermore, the defective intracellular replication of the polyamine mutants with *speB*, *speC*, *speE*, and *speF* deletions was in concordance with the downregulation of SPI-1 and SPI-2 genes in *S. Typhimurium* (Jelsbak et al., 2012). Consistently, our microarray study demonstrated the significant downregulation of SPI-1 genes (e.g., *sopD*, *sopA*, and *sopE2*) and SPI-2 genes (e.g., *sseA*, *ssah*, *sseB*, and *ssaG*) through *speG* deletion in *S. Typhimurium* (Table 2B). Altogether, polyamines are required for the induction of SPI-1 and SPI-2 for *Salmonella* flagellation and motility, and they function as an environmental stimulus to prime *S. Typhimurium* for intracellular proliferation in human epithelial cells.

*speG* can significantly regulate the expression of different genes before and after the internalization of *S. Typhimurium* in human intestinal epithelial cells. For *S. Typhimurium* outside host cells, *speG* is required for the expression of the well-documented genes encoding flagella, fimbra, and T3SS, as well as for the expression of 66 other genes, including those involved in the biosynthesis of inner and outer membranes, bacteriophages, and hydrogenase-1 operon proteins and a few genes encoding hypothetical proteins (Table 2B). Moreover, *speG* can suppress the upregulation of several groups of genes involved in the periplasmic nitrate reductase system (*yofF* and *napF*), glucarate metabolism (e.g., *garL* and *garR*), the phosphotransferase system (SL1344_3736 and SL1344_4467), cytochromes (*cyoABC*), and the succinate reductase complex (*sdhABCD*; Table 2A). These genes have rarely been reported to correlate with the polyamine metabolism and intracellular replication of *Salmonella*. The nap genes encoding periplasmic nitrate reductase can be upregulated under low nitrate conditions from aerobic to anaerobic metabolism to contribute to the luminal growth and virulence of *Salmonella in vivo* (Paiva et al., 2009; Rowley et al., 2012; Lopez et al., 2015). Meanwhile, *speG* could be involved in carbon metabolism for energy because *garL* and *garR* correlate with hydrogen-stimulated carbon acquisition for hydrogen-dependent growth (Lamichhane-Khadka et al., 2011). *speG* modulates SPI-1 virulence because phosphotransferase controls the global transcription regulator Mlc for the complete expression of hilA, hilD, and invF (Poncet et al., 2009). Similar to the upregulation of *cyoA*, *cyoB*, and *cyoC* after *speG* deletion in *S. Typhimurium* in our study (Result 3.6), cytochrome *bo* oxidase genes (*cyoA*, *cyoB*, and *cyoC*) were significantly upregulated on exposure to nitrogen oxide stress, which reduces their vulnerability to oxidative injury (Calderon et al., 2014). Therefore, without *speG* expression, *S. Typhimurium* tends to lose protection against oxidative injury by activating cytochrome *bo* oxidase. In our study, *speG* significantly regulated the expression of four genes encoding the four subunits of the succinate dehydrogenase complex, which is the only enzyme participating in both the electron transport chain and TCA cycle (Oyedotun and Lemire, 2004). Altogether, *speG* can influence a broad spectrum of bacterial metabolisms involving reduction, oxidation, and energy consumption in addition to influencing known virulence factors. In Caco-2 cells, the *ilv-leu* operon (*ilvA*, *ilvC*, *ilvG*, *ilvM*, *leuB*, *leuC*, and *leuD*) and the histidine operon (*hisC* and *hisG*) were significantly upregulated after *speG* deletion in intracellular *S. Typhimurium* (Table 3A), suggesting the inhibitory function of both operons in the intracellular replication of *Salmonella*. Mutations in *hisG* trigger the intracellular filamentous growth of *S. Typhimurium* and bacterial cell division in eukaryotic host cells (Henry et al., 2005), which is in concordance with our observation of *hisG* upregulation in the poorly proliferating *speG* mutant of intracellular *S. Typhimurium* in Caco-2 cells.

In conclusion, *speG* is required for the intracellular replication of *S. Typhimurium* and is dependent on the duration after bacterial invasion and independent on cell types. Putrescine, spermine, and cadaverine are the predominant polyamines in *S. Typhimurium*. The effects of *speG* on the biosynthesis of these polyamines vary outside and inside host cells and affect the expression of previously reported and unreported *Salmonella* virulence genes involved in a broad spectrum of cellular functions rather than other polyamine-associated genes. Thus, *speG* plays an independent key role in the polyamine metabolism and virulence regulation of *Salmonella*.

**ETHICS STATEMENT**

This study was approved by Taipei Medical University-Joint Institutional Review Board (No. 201205007). Neither human participants nor animals were involved in this study.

**AUTHOR CONTRIBUTIONS**

S-BF designed the research and wrote the article; C-JH, C-HH, K-CW, N-WC, and H-YP performed the experiments and contributed reagents, materials, and analysis tools. S-BF designed the research and wrote the article; C-JH, C-HH, K-CW, N-WC, and H-YP performed the experiments and contributed reagents, materials, and analysis tools.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02245/full#supplementary-material

**Supplementary Figure 1** | The HPLC chromatogram of the mixed four diluted polyamine standards.

**Supplementary Figure 2** | The regression equations derived from the five dilutions of four polyamine standards.
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