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Shuhui li

SALMONELLA ENTERICA SEROVAR ENTERITIDIS REQUIRES THE TYPE THREE SECRETION SYSTEM-1/2 TO INVADE/SURVIVE IN CHICKEN OVIDUCT EPITHELIAL CELLS AND TO MODULATE INNATE IMMUNE RESPONSES

By

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Contaminated poultry and egg products are major sources of *Salmonella enterica* serovar Enteritidis (*S. enteritidis*, SE) infections in humans. Colonization of SE in chicken reproductive tract results in the production of contaminated commercial shell-eggs and fertilized hatchery eggs. The complex pathogen-host interactions during SE colonization of chicken reproductive tract are largely unknown. This study was aimed at determining the pathogenic roles of the type three secretion systems (TTSS-1 and TTSS-2) in SE infection of chicken oviduct epithelial cells (COEC). A series of SE strains carrying mutations in the genes encoding structure or effector proteins of TTSS-1 and TTSS-2 were constructed. The invasiveness and intracellular survival rate of each SE strain as well as the host innate immune responses induced by the infections were evaluated. The results demonstrate that both TTSS-1 and TTSS-2 are required by SE to invade COEC which involve genes encoding effector proteins

SipA, SopB, SopE2, and PipB. In addition to their involvement in host cell invasion, *sipA* and *sipB* are also necessary for the survival or replication of SE inside COEC. Inactivation of TTSS-2 genes (*ssaV* and *pipB*) resulted in an enhanced bacterial proliferation inside COEC. The data from this study also show that SE infection triggers pro-inflammatory responses in COEC and TTSS-1 is involved in the expression of iNOS and IL-8, a CXC chemokine. TTSS-1 and TTSS-2 are not necessary for induction of K203, MIP-1β, and IL-10 or suppression of TGF-β3 in COEC.

DEDICATION

I would like to dedicate this research to my parents, Guoxing Li and Yuhuan Zhang, and my husband Changyou Lin.

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SECTION Ⅰ

INTRODUCTION

Salmonella enterica serovar Enteritidis (*S. enteritidis,* SE)-induced enterocolitis is one of the most important human bacterial diseases. *S. enteritidis* isolated from human sources ranks second among all the *Salmonella enterica*, after serovar Typhimurium (*S. typhimurium*) [1, 2]. Chicken and chicken products are the major sources of *S. enteritidis* infection. In 2005, *S. enteritidis* was the most common serovar, as 67 out of 195 cases, isolated from the chicken [2]. Grade A eggs had once been considered as a major source of *S. enteritidis* infection in humans from the1980's to the early 1990's [3-9]. The number of *S. enteritidis* isolates reported to CDC reached the peak at 3.8 per 100,000 population in 1995 [10]. To ease this important public health problem, stringent control measures have been implemented by the US Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS), one of which is testing *S. enteritidis* in broiler chicken carcass rinse collections. These establishments have covered more than 95% of raw chicken market in the US. Though the incidence of *S. enteritidis* infection in human decreased greatly after 1996, the number of *S. enteritidis* contaminated broiler carcass rinses increased more than 4-fold annually from 2000 through 2005. Therefore, *S. enteritidis* outbreaks

associated with poultry products remain a public health concern [10-12].

Among the 2,300 plus serovars of *Salmonella enterica*, *S. enteritidis* infect adult chickens without causing overt clinical signs and infected laying hens may produce normal-appearing eggs contaminated with *S. enteritidis*. Although previous research has demonstrated that the tubular glands of the isthmus are the predominant site of *S. enteritidis* infection in chicken reproductive tract, limited information regarding the molecular mechanisms by which *S. enteritidis* colonizes chicken reproductive tract is available [13].

The virulence of *Salmonella enterica* is essentially associated with two Type Three Secretion Systems (TTSSs) that are located in two large genomic elements, called *Salmonella* pathogenicity island-1 and -2 (SPI-1 and SPI-2) [14-16]. The structural proteins of TTSS form a needle-like complex that crosses through both the inner and outer membranes of the bacterial envelope and closely resembles the flagella basal body [17]. Both TTSS-1 and TTSS-2 have evolved to deliver effector proteins from the bacterial cytoplasm into host cell cytosol to initiate bacteria-host interactions and modulate host cellular functions. The TTSS effector proteins may be encoded by genes located within SPI-1 and SPI-2, or another pathogenicity island, namely SPI-5 [18-20]. In general, TTSS-1 is highly expressed in rich culture broth at the mid-log phase of the growth [21], and is responsible for host cell invasion and modulation of host immune responses [22-30]. After bacterial internalization, acidified vacuoles, termed the *Salmonella*-containing vacuoles (SCVs), are formed, and bacteria reside within the SCV. The expression of TTSS-2 is induced in SCV or in

medium with low concentrations of magnesium and calcium and low pH resembling the environmental conditions of SCV [31]. TTSS-2 is required for the intracellular survival and replication of *Salmonella* in host macrophages as well as the establishment of systemic infections [15, 32].

The TTSS-1 requires several *Salmonella* invasion proteins (Sips), including SipB/ *Salmonella* secreted proteins B (SspB), SipC (SspC) and SipD (SspD), to form the translocation complex. Inactivation of SipB, SipC or SipD renders *Salmonella* unable to deliver effector proteins into host cell cytosol, resulting in an invasion defect [33]. The effector proteins translocated by TTSS-1, such as SipA, *Salmonella* outer protein B (SopB), SopE, and SopE2 are very important in the entry *Salmonella* into host cells in mammalian system [34]. SipA (SspA) encoded by SPI-1 [35] is a homologues of Invasion plasmid antigen A (IpaA), a Shigella protein necessary for host cell invasion [36, 37]. *Salmonella* outer protein B (SopB)/ *Salmonella* invasion gene (SigD) encoded by SPI-5 is a *Salmonella* homologue of Invasion plasmid gene (IpgD) from *Shigella* [38]. SopE2 is encoded by a gene located in the centisome 40-42, which is 69% identical to SopE [39]. SopB is an inositol polyphosphate phosphatase, which indirectly stimulate Cdc42 signaling [40-42]. SopE2 acts as G-nucleotide exchange factors (GEFs) to activate Rho GTPase Cdc42. Activation of Cdc42 by SopB or SopE2 triggers the host cell actin cytoskeleton rearrangements, macropinocytosis and bacterial internalization [43]. SipA lowers the critical concentration of G-actin and increases the stability of F-actin at the site of bacterial entry which facilitates invasion [24]. Inactivation of *sopB* or *sopE2* results in a significant reduction in the invasiveness of *S. typhimurium*.

In contrast, a mutation in the *sipA* gene causes only a brief delay (5-min) in *S. typhimurium* invasion of mammalian cells and the *sipA* mutant is as invasive as its wild type parent strain when bacteria are recovered at 15 min following inoculation [25].

In addition to their involvement in host cell invasion, the effector proteins of TTSS-1 may also participate in the late stages of *S. typhimurium* infection in mice, because these three proteins were continually expressed for several days in the colonized sites [44]. Furthermore, these proteins contribute to *Salmonella* pathogenesis by modulating host immune responses. It has been previously shown that SipB, a translocon and an effector of TTSS-1, triggers the secretion of inflammatory cytokine interlukin-1β (IL-1β) [26] and inducible nitric oxide synthase synthesis [28]. The effector proteins, SipA, SopB, and SopE2 induce the production of IL-8 in infected epithelial cells, the expression of iNOS in *Salmonella*-infected macrophages, and polymorphonuclear leukocytes (PMNs) influx and fluid accumulation in bovine ligated ileal loops [28, 45]. SipA is sufficient to elicit the transepithelial PMNs migration in tissue culture model [46]. While SipB induces apoptosis in macrophage by directly targeting to host cell caspase-1, SopB has an anti-apoptotic activity in *Salmonella* infected epithelial cells by sustained activation of Akt [26, 27].

The components of TTSS-2 secretion system apparatus (*ssa*) are encoded by 13 genes located at 30 centisomes on the *Salmonella* chromosome, including *ssaJ-M*, *ssaV*, *ssaN-T,* and *ssaU*. Mutations in genes abolishes the ability of TTSS-2 to

translocate its effector proteins into host cells, thereby resulting in significantly attenuated phenotypes, in terms of intracellular replication in macrophages and establishment of systemic infections, although the degree of attenuation may vary depending on which gene is inactivated [47]. As a structural protein of TTSS-2, SsaV is integral to the inner bacterial cell membranes and is also required for *Salmonella* induced delayed cytotoxicity of human macrophages [48]. Because of the attenuated phenotype conferred by inactivation of the *ssaV* gene, attempts have been made to use *ssaV* mutants as typhoid vaccines and delivering vectors for antigens of other pathogens [49-51]. Several other genes are also required for the function of TTSS-2, such as *ssr* (secretion system regulator) and *ssc* (secretion system chaperone) genes that encode the regulators and secretion chaperones, respectively. The proteins coded by the *sse* (secretion system effector) genes serve as the effectors of TTSS-2 [47, 52]. Similar to TTSS-1, certain effector genes of TTSS-2 are located outside SPI-2, such as pathogenicity island encoded protein B (PipB) which is encoded by a gene in SPI-5 [19]. The *pipB* gene is not present in all the *Salmonella* spp, such as *S. bongaris*. The expression of *pipB* is regulated by the other SPI-2 proteins, including SsaM and SpiC, and occurs following *Salmonella* invasion of mammalian host cells [53]. PipB and its homologue, PipB2, are translocated by TTSS-2 to the S*almonella*-containing vacuoles (SCV) and *Salmonella*-induced filaments (Sifs). Although PipB2 mediates the extension of Sif via the biological activity of its C-terminal peptapeptide motif, the exact function of PipB remains to be investigated [54]. Much of our understanding on TTSS-1 and TTSS-2 is based on the investigations involving mammalian infection

models. Recent studies suggest that both TTSS-1 and TTSS-2 play essential roles in the pathogenesis of *Salmonella enterica* in chicken hosts. However, the exact contributions of individual effector proteins of TTSS-1/2 to *S. enteritidis* colonization of chicken hosts remains to be determined [55].

The outcome of an infection is determined by the complex interplay between a pathogen and its host. It's known that the innate immune response is the first line of defense that limits the pathogen locally. In mammalian systems, the innate host immune responses to *Salmonella* infection have been studied extensively. For example, the SPI-1 encoded TTSS-1 has been shown to play an important role in the induction of the inflammatory response and fluid accumulation in bovine intestine [56, 57]. Recent progress in determination of cytokine and chemokine sequences of avian hosts has made it possible to assess chicken innate immune responses following *Salmonella* infection in chickens or chicken tissue cultures. Data from recent studies indicate that differential immune responses are elicited by different *Salmonella spp. S. gallinarum* infection does not induce a strong inflammatory response in local sites, resulting in a severe systemic disease. In contrast, *S. typhimurium* and *S. enteritidis* trigger a strong innate immune response contributing to the clearance of bacteria in the intestine and consequently less severe systemic infection [58]. Up to the present, most of the studies have been focused on determining the expression profiles of cytokines/chemokines in macrophages, spleen, liver, and intestine of chicks, possibly due to the convenience of the experimental system [59-62]. No investigation has been carried out to determine the innate immune responses in chicken reproductive tissues

following infection with *S. enteritidis*.

The present study is aimed at understanding the pathogenic roles of TTSS-1 and TTSS-2 in *S. enteritidis* infection of primary chicken oviduct epithelial cells (COEC). We have determined the contributions of individual effector proteins of TTSS-1 and TTSS-2 in the entry of *S. enteritidis* into COEC and subsequent intracellular survival and replication of this organism. In addition, we have assessed the role of TTSS effectors in induction of pro- and anti-inflammatory cytokines and chemokines in infected COEC.

SECTION Ⅱ

MATERIAL AND METHODS

2.1 Bacterial strains and growth conditions.

S. enteritidis and *E. coli* strains used in this study are listed in Table1. All the bacteria were cultured at 37℃ in Luria Bertani (LB) agar plates or Trypticase Soy Broth (TSB), supplemented with appropriate antibiotics at the following concentrations: chloramphenicol, 30μg ml^{-1}; ampicillin, 100μg ml^{-1}; nalidixic acid, 50μg ml^{-1}.

2.2 Construction of mutants and genetic complementation.

Strains ZM101 (*sipB*), ZM102 (*ssaV*), ZM103 (*sipA*), ZM104 (*sopB*), ZM105 (*sopE2*), and ZM106 (*pipB*) were constructed by allelic exchange. In brief, the primer pairs sipBF and sipBB, ssaVF plus ssaVB, sipAF plus sipAB, sopBF plus sopBB, sopE2F plus sopE2B, and pipBF plus pipBB were used to amplify the internal fragment target genes, including *ssaV*, *sipA*, *sopB*, *sopE2*, and *pipB* (Table 2). The PCR products were cloned into vector pCR2.1TOPO (Invitrogen) according to the manufacture's instructions. The inserts were excised from pCR2.1TOPO by X*ba*I and S*ac*I digestion and subcloned into the corresponding sites of pEP185.2, a suicide

vector coding for chloramphenicol resistance. The genetic organization of T3SS genes involved in host cell invasion or intracellular survival of SE is shown in Figure 1. The resulting plasmids, pZM101, pZM102, pZM103, pZM104, pZM105, and pZM106, as listed in Table 1, were introduced into *E. coli* strain S17λ-*pir* by chemical transformation and then transferred into SE strain ZM100 by conjugation. The exconjugants in which the suicide plasmid was integrated into the ZM100 chromosome were selected LB-Agar supplemented with nalidixic acid and chloramphenicol. Insertions of the suicide vector in the target genes of mutant strains were confirmed by Southern hybridization analyses. In brief, an internal fragment of each gene was amplified by PCR, purified with phenol and chloroform, and labeled with a thermostable alkaline phosphatase enzyme using the AlkPhos Direct Labelling Reagents with CDP-*Star* Kit. The labeled PCR products were used as probes for Southern hybridization. Chromosomal DNA of each strain was extracted using the CTAB method. Following restriction digestion with enzyme P*st* Ⅰ, the chromosomal DNA was subjected to gel eletrophoresis, transferred onto a positive charged nylon membrane, and hybridized to an appropriate probe. Hybridization and subsequent detection using CDP-*Star* detection reagent were performed according to the manufacture's instructions (GE Healthcare).

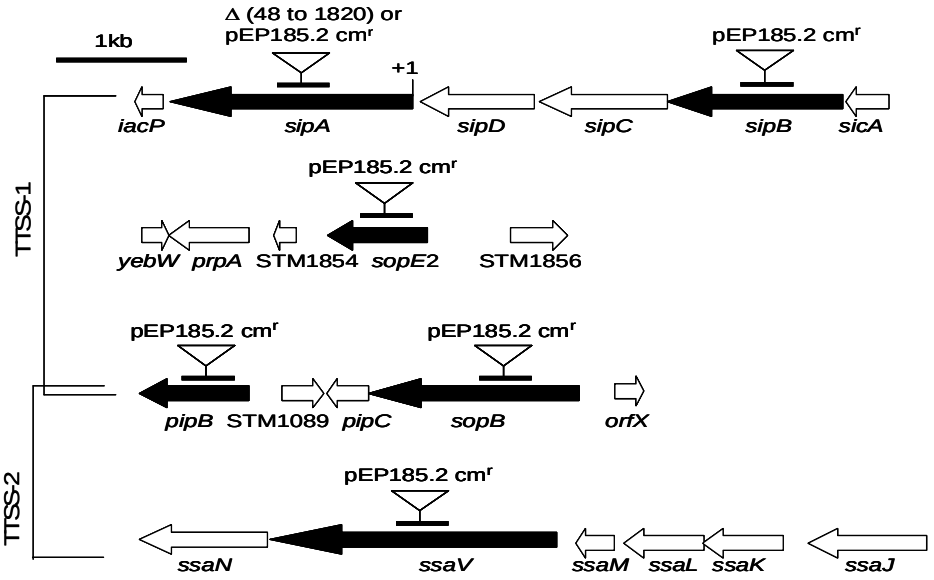

Figure 1 The genetic organization of T3SS genes involved in host cell invasion or intracellular survival of SE.

Note: Mutations in TTSS genes (solid arrows) required for host cell invasion or intracellular survival of SE. The open arrows indicate genes in the surrounding regions. The bars indicate internal fragments of *sipA*, *sipB*, *sopB*, *sopE2*, *ssaV*, and *pipB* that were cloned into pEP185.2 to inactivate these genes. Numbers indicate the position of deletion relative to the first nucleotide (+1) in the open reading frame of *sipA*.

For genetic complementation, the entire open reading frame (ORF) of *sopB*, *sopE2*, and *pipB* were amplified by PCR using primers sopBcomF plus sopBcomB, sopE2comF plus sopE2comB, and pipBcomF plus pipBcomB. The PCR products were cloned into cloning vector pCR2.1 TOPO (Invitrogen), and then subcloned into directionally behind the *lac* promoter of pWSK29, a low-copy-number expression vector, to generate plasmids pZM104-c, pZM105-c and pZM106-c. The sequence of each insert and its correct orientation was confirmed by DNA sequencing performed by MWG. Plasmid pZM103-c, pZM104-c, pZM105-c, and pZM106-c were introduced into the corresponding mutants ZM103, ZM104, ZM105, and ZM106 to

generate complemented strains ZM103-c, ZM104-c, ZM105-c, and ZM106-c, respectively. Transformations of mutant strains were carried out by electroporation and subsequent selection for resistance to ampicillin. The complemented SE strains are listed in table 1.

Table 1 Bacterial Strains used in this study

Strains	Relevant genotype	Source or reference
S. enteritidis		
SE338	Clinical isolation	
ZM100	Derivative of SE338, nal^r, DT4	This study
ZM101	Derivative of SE100, Δ*sipB*, nal^r cm^r	This study
ZM102	Derivative of SE100, Δ*ssaV*, nal^r, cm^r	This study
ZM103	Derivative of SE100, Δ*sipA*, nal^r, cm^r	This study
ZM104	Derivative of SE100, Δ*sopB*, nal^r , cm^r	This study
ZM105	Derivative of SE100, Δ*sopE2*, nal^r, cm^r	This study
ZM106	Derivative of SE100, Δ*pipB* , nal^r, cm^r	This study
ZM103-c	Derivative of SE103, carrying pZM103-c, nal^r, cm^r,amp^r	This study
ZM104-c	Derivative of SE104, carrying pZM104-c, nal^r, cm^r,amp^r	This study
ZM105-c	Derivative of SE105, carrying pZM105-c, nal^r, cm^r,amp^r	This study
ZM106-c	Derivative of SE106, carrying pZM106-c, nal^r, cm^r,amp^r	This study
E. coli		
S17 λ *-pir*	π protein; RP4	

Table 2 Primers used to amplify TTSS genes

Gene	Forward primer sequence (F)	Backward primer sequence (B)	Size of Amplicon
ssaV	GCATCTAGAGTCACTCACAATCAGCACA	GCACATATCCCGCGAAAACGTCCAGTCC	1127
sipA	ATGGTTACAAGTGTAAGG	CAGCCAAAGTTATGTTCA	590
sopB	CCCGTATTTGGTTCTGAATCTCC	AGCCTGAAACTGGTATCCGTGC	677
sopE	GCTTCTGAGGGTAGGGCGGTATTA	GTTGTGGCGTTGGCATCGTC	457
pipB	ACGCGGTATACTGGAATGGTTT	TGCGTGAGTCAGGTTTGCTTTAGT	561

Table 3 Plasmids used to construct mutant SE strains

Plasmids	Relevant genotype	Source or reference
pCR2.1	*Amp*^{r}, *Kan*^{r}, *lacZ* α	Invitrogen
pEP185.2	*Cm*^{r}	
pZM101	pEP185.2 carrying the internal region of *sipB*	SZ lab stock
pZM102	pEP185.2 carrying the internal region of *ssaV*	This study
pZM103	pEP185.2 carrying the internal region of *sipA*	This study
pZM104	pEP185.2 carrying the internal region of so*pB*	This study
pZM105	pEP185.2 carrying the internal region of *sopE*	This study
pZM106	pEP185.2 carrying the internal region of *pipB*	This study

Table 4 Primers used for genetic complementation

Name of Amplicon	Forward primer sequence (F)	Backward primer sequence (B)	Size of Amplicon
sopBcom	GCGAGCTCATGATCGCCACTACGTATG	GCTCTAGATCAAGATGTGATTAATGAAG	1858
sopE2com	GCGAGCTCGTGACAGAAGAACAAAATCC	GCTCTAGATCAGGAGGCATTCTGAAGAT	868
pipBcom	GCGAGCTCCTTTAAGTAAATTTTCGCTC	GCTCTAGACTAAAATCTCGGATGGGGGG	1178

Note: The restriction endonuclease sites incorporated into the primer sequences are underlined.

Table 5 Plasmids used for genetic complementation

Name of Plasmids	Relevant genotype	Source or reference
pWSK29	*Amp*^{r}	[63]
pZM103-c	pWSK29 carrying the whole ORF of *sipA*	[56]
pZM104-c	pWSK29 carrying the whole ORF of *sopB*	This study
pZM105-c	pWSK29 carrying the whole ORF of *sopE2*	This study
pZM106-c	pWSK29 carrying the whole ORF of *pipB*	This study

2.3 Infection of chicken oviduct epithelial cells (COEC)

Chicken oviduct epithelial cell cultures**.** The oviduct tissues of 25-28 week old broiler breeder hens were donated by a commercial poultry that wishes to remain anonymous. *Salmonella*-free status of the hens was confirmed by performing a series

of bacterial cultures. Upon receiving the tissues, the isthmus region of the oviduct was dissected and washed extensively with HBSS containing 200 U ml^{-1} penicillin and 200 mg ml^{-1} streptomycin. The tissues were cut into small pieces and digested with collagenase at 37℃ for 20 and 30 minutes and then treated with Trypsin-EDTA (Gibco Invitrogen) three times at 37℃ for 15 minutes with occasional shaking. The supernatants containing digested chicken oviduct epithelial cells (COEC) were collected and mixed with equal volume of MEM (ATCC) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2% chicken serum (CS), insulin (0.12U ml^{-1}), and estradiol (50 nM). The cell mixtures were combined together, and filtered through cell strainers with pore size of 100 μM and then centrifuged at 50g for 5 minutes at 4℃ to remove tissue clumps and contaminating erythrocytes, respectively. The COEC pellets were resuspended in fresh MEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2% chicken serum (CS), insulin (0.12U ml^{-1}), and estradiol (50 nM), and seeded into 96-well tissue culture plates at a density of 2 x 10^4 cells per well in 0.2ml medium. The COEC cultures were incubated at 39℃ in 5% CO2 for 24-48 hours till a confluent monolayer was obtained. [13]

Invasion and intracellular survival assays. Wild type SE, ZM100, was grown in Trypticase Soy Broth (TSB) supplemented with 50μg ml^{-1} nalidixic acid. All mutant SE organisms were cultured in TSB supplemented with 50μg ml^{-1} nalidixic acid and 30μg ml^{-1} chloramphenicol. The overnight aerobic cultures of SE strains were diluted in fresh TSB (1:50X) supplemented with appropriate antibiotics and then incubated aerobically at 37℃ for 4 hours to reach logarithmic phase of growth. The number of

bacteria in each culture was determined based on the OD600 measurement obtained using a Thermo Scientific* Spectronic* BioMate 3 Spectrophotometer. The appropriate amount of bacterial culture was centrifuged at 11,000g for 5 minutes and the bacterial pellet was resuspended in invasion media (MEM with insulin and estrogen, and without antibiotics, see appendix). The bacterial suspension was inoculated onto the COEC monolayer at a multiplicity of infection (MOI) of 20:1. For each bacterial strain, 6 wells of COEC cultures were included, of which 3 wells were used for recovery of intracellular bacteria and the remaining 3 wells were used for RNA extraction. The inoculated COEC cultures were centrifuged at 800g for 5 min to synchronize the infection and then incubated at 39℃ in 5% CO_2 for 1 hour. Following incubation, the infected COEC cultures were washed 3 times with plain MEM and incubated in MEM culture medium containing 100 μg ml^{-1} gentamicin at 39℃ in 5% CO_2 for 1 hour to kill extracellular bacteria. After gentamicin treatment, the COEC cultures were washed 3 times with plain MEM and either harvested for recovery of intracellular bacteria and RNA extraction or maintained in complete MEM for additional 4 and 24 hours. Upon completion of incubations, the infected COEC cultures were lysed with 0.05% Triton-X100 (Sigma) and the numbers of intracellular bacteria at 1, 4, and 24 hours post infection (T1, T4, and T24) were determined by plating serial dilutions of lysed COEC cultures on LB agar plates supplemented with 50μg ml^{-1} nalidixic acid or chroramphenicol. The number of bacteria in each inoculum was also confirmed by plating a portion of serial dilutions on LB agar plates. Invasiveness of each strain was expressed as percent of inoculum

by dividing the number of intracellular bacteria with the number of bacteria in inoculum, whereas the survival or replication rates were presented as fold-change in the number of intracellular bacteria at T4 and T24 over T1 [13, 55, 64, 65]

2.4 Determination of cytokine or chemokine expression in COEC by reverse transcription real-time PCR

The expression of cytokines and chemokines induced by SE strains in COEC was determined by two-step RT-PCR. Total RNA was extracted from COEC lysates from triplicate wells using Trizol reagents according to the manufacture's instruction. Reverse transcription was performed using a TaqMan Reverse Transcription Reagents Kit from Applied Biosystems according to the manufacturer's instruction. Real-time PCR (RT-PCR) was carried out for each cDNA sample in duplicate by using gene-specific primers (0.8μM final concentration) and Applied Biosystems SYBR Green I PCR master mix kit according to the manufacture's instruction. All primers used for RT-PCR reactions were listed in Table 6. PCR reactions were performed on an Applied Biosystems 7000 Real-time PCR System instrument (Applied Biosystems) according to the manufacturer's instructions. The threshold cycle (Ct) value was determined for each sample and mRNA concentration for each target gene was quantified using the comparative Ct method [66]. Real-time amplification of β-actin was used to normalize the cDNA concentration of different samples with the assumption that the expression of β-actin remains unchanged during infection. The normalized amount of transcripts relative to the amount of transcripts present in

samples from an uninfected control loop was given as $2^{-\Delta\Delta Ct \pm S}$ (fold-change) where S is the standard deviation.

Table 6 Primers for real-time PCR

Name of Amplicon	Forward primer sequence (F)	Backward primer sequence (B)	Size of Amplicon
β-Actin	TGCGTGACATCAAGGAGAAG	GACCATCAGGGAGTTCATAGC	111
iNOS	AGTTTGAAATCCAGTCGTGTTAC	AATATGTTCTCCAGGCAGGTAG	87
IL-8	GTAGGACGCTGGTAAAGATGG	TAGGGTGGATGAACTTAGAATGAG	75
MIP-1β	CAGACTACTACGAGACCAACAG	GCATCAGTTCAGTTCCATCTTG	137
K203	TCTTCTCATCGCATCCTTCTG	ATCTTGTGTGTTATGTAAGTGGTG	93
IL-10	AGCCATCAAGCAGATCAAGG	ACTTCCTCCTCCTCATCAGC	125
TGFβ3	ACGACACAAAGACCACACTC	CATTCAGATACACAGCAGTTCC	84

2.5. Statistical analysis.

The mean value of data collected from at least three independent experiments and the standard deviation were calculated by using SAS9.1 software. The differences in invasiveness and intracellular survival/replication rate between each mutant and wild type strain, ZM100, were analyzed using Student-*t* test. The differences in the expression levels of cytokines and chemokines among COEC cultured infected with wild type and mutant SE strains were determined using the SAS 9.1 software with *t* test (LSD) program.

SECTION Ⅲ

RESULTS

3.1. Contributions of TTSS-1 and TTSS-2 to SE invasion of primary COEC.

The contribution of TTSS-1 and TTSS-2 to SE invasion of primary COEC was assessed using a standard gentamicin protection assay [13, 55, 64, 65]. The TTSS-1 null mutant, ZM101 (*sipB*⁻) was approximately 1-log less invasive than the wild type strain, SE100 (Figure 2). Strains harboring insertion mutations in TTSS-1 effector genes, including *sipA*, *sopB*, and *sopE2*, exhibited about 2-fold reduction in invasiveness, compared to ZM100 (*wt*). In addition, the TTSS-2 mutants ZM102 and ZM106 carrying mutations in genes encoding a structural protein, SsaV, and an effector protein, PipB, showed 41.99% and 31.78% reduction in invasiveness, respectively, compared to that of the wild type strain ZM100. In contrast to previous findings that a brief delay in serovar Typhimurium invasion of mammalian epithelial cells was caused by inactivation of *sipA*, the decreased invasiveness of ZM103 was statistically significant at 1hpi of COEC. These data provide clear evidence that the effector proteins of TTSS-1 (SipA, SopB and SopE2) and TTSS-2 (PipB) are equally important in the entry of SE into COEC.

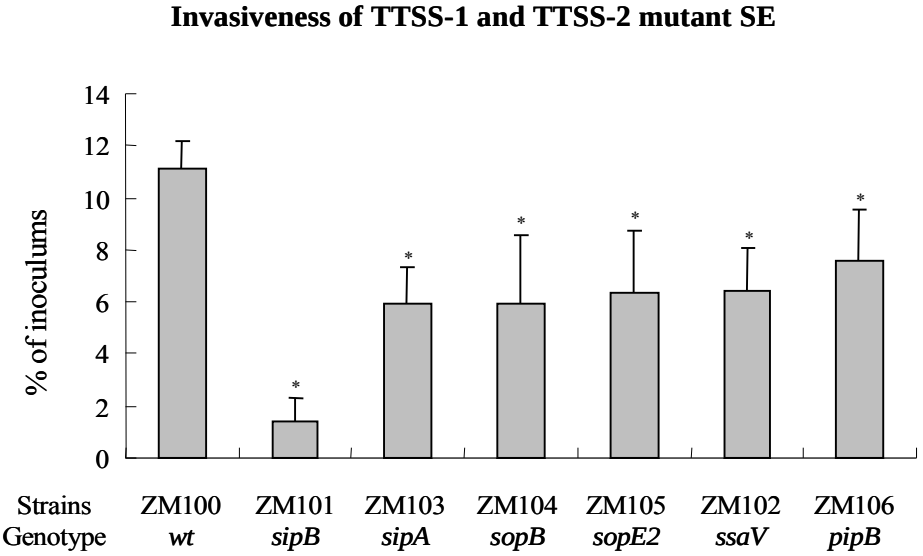

Figure 2 Invasiveness of SE strains was determined by gentamicin protection assays.

Note: Data shown (bars) are geometric means of three independent experiments ± standard deviation. Asterisks indicate that the differences between the invasiveness of mutants and that of the wild type SE are statistically significant ($p < 0.01$). wt, wild type.

3.2. Roles of TTSS-1 and TTSS-2 in the intracellular survival and replication of SE in COEC.

To evaluate the roles of TTSS-1 and TTSS-2 in the survival or replication of SE inside COEC, the ratio between the numbers of intracellular bacteria of each strain at 4hpi and 1hpi or 24hpi and 1hpiwas determined and designated as the net survival/replication rate. At 4hpi, the TTSS-1 null mutant, SE101 (*sipB*$^-$), showed a significant reduction in the net survival inside COEC (Figure 3). Inactivation of a TTSS-1 effector gene, *sipA,* also resulted in a significantly decreased survival. Although net survival/replication of ZM104 (*sopB*$^-$) was less than that of ZM100 (wt), the difference was not statistically significant. Unlike TTSS-1 mutants, ZM102 (*ssaV*$^-$)

and ZM106 (*pipB*$^-$) showed a slightly increased ability, not statistically significant, to proliferate inside COEC. Of the five TTSS-1/2 effector genes examined, *sopE2* had the least influence on the ability of SE to survive or replicate in COEC, although it was previously identified as a colonization factor of serovar Typhimurium in chick intestine [34, 39]. At 24hpi, the survival of ZM101 (*sipB*$^-$) was still significantly lower than that of the wild type strain, ZM100 ($P < 0.05$), whereas the net survival of ZM103, although sharply reduced, was not significantly different from that of ZM100 due to experimental variations. The *sopB* and *sopE*2 mutants displayed similar survival rates to that of ZM100. In contrast, the two TTSS-2 mutants, namely ZM102 (*ssaV*$^-$) and ZM106 (*pipB*$^-$), displayed significantly higher replication rates than ZM100 ($P < 0.05$) (Figure 4).

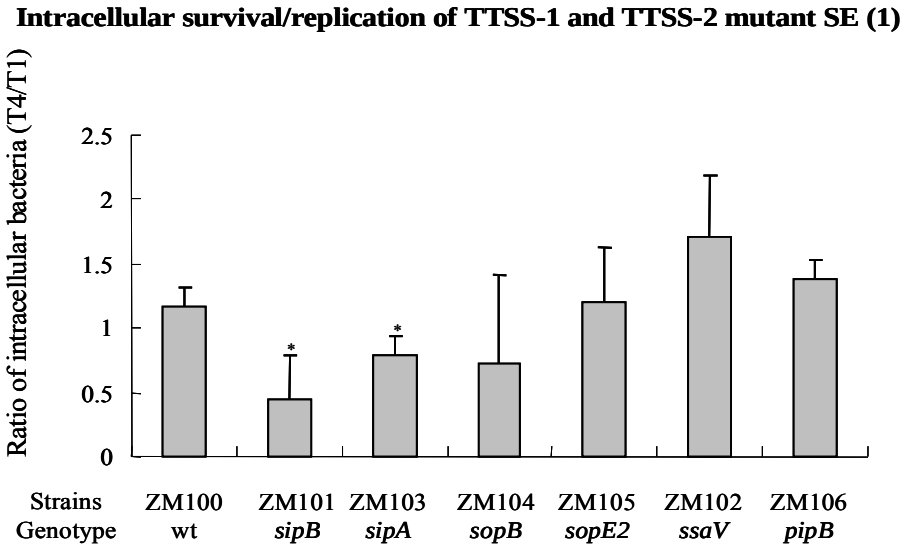

Figure 3 Intracellular survival/replication of TTSS-1 and TTSS-2 mutant SE (1).

Note: The net survival/replication is presented as ratio between the numbers of intracellular bacteria recovered at T4 and T1. Data shown (bars) are geometric means of three independent experiments ±

standard deviation. Asterisks indicate that the difference between the net survival/replication mutants and that of the wild type strain are statistically significant ($P < 0.05$). wt, wild type.

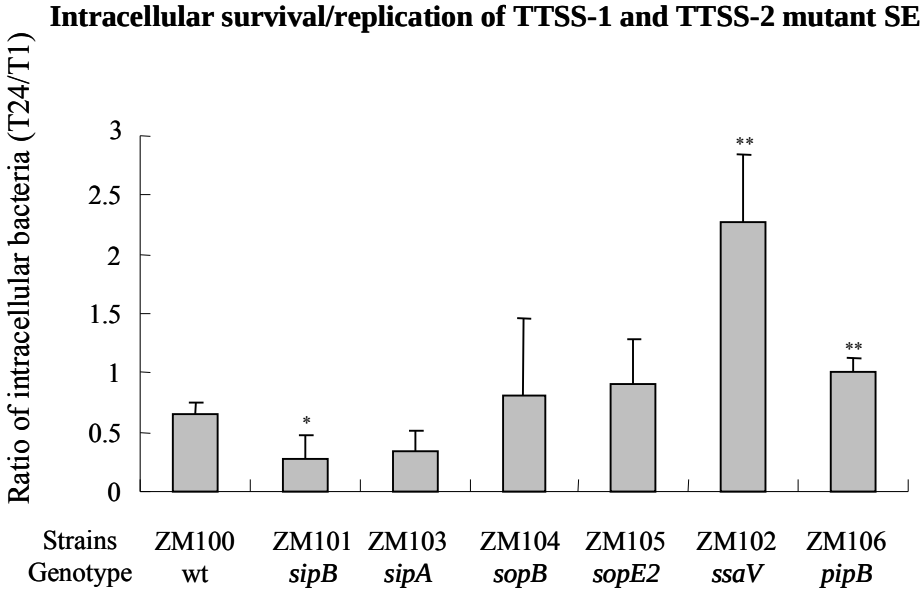

Figure 4 Intracellular survival/replication of TTSS-1 and TTSS-2 mutant SE (2).

Note: The net survival/replication is presented as ratio between the numbers of intracellular bacteria recovered at T24 and T1. Data shown (bars) are geometric means of three independent experiments ± standard deviation. Single asterisk indicates that the net survival of ZM101 was significantly lower than that of the wild type strain ($P < 0.05$). Double asterisks indicate that the net survival of ZM102 or ZM106 was significantly higher than that of the wild type strain ($P < 0.05$). wt, wild type.

3.3. Genetic complementation to confirm the contributions of TTSS-1/2 to SE invasion and survival in COEC.

We next conducted a series of genetic complementation assays to confirm the pathogenic roles of TTSS-1 and TTSS-2 effectors in the entry of SE into COEC. The invasiveness of each complemented strain to invade COEC was compared to that of the wild type strain ZM100 and its isogenic parent strain. Our results demonstrated that the complemented strains were significantly more invasive than their parent

strains, but not the wild type SE, ZM100. Thus, it is apparent that the invasion defects associated with mutations in individual effector genes, including *sipA*, *sopB*, *sopE2,* and *pipB* were fully restored by introducing the cloned genes on plasmids (pZM103-c, pZM104-c, pZM105-c, and pZM106-c) into the corresponding isogenic parent strains (Figure 5). These data clearly suggest that the entry of SE into chicken oviduct epithelium requires the effector proteins translocated by both virulence-associated TTSSs.

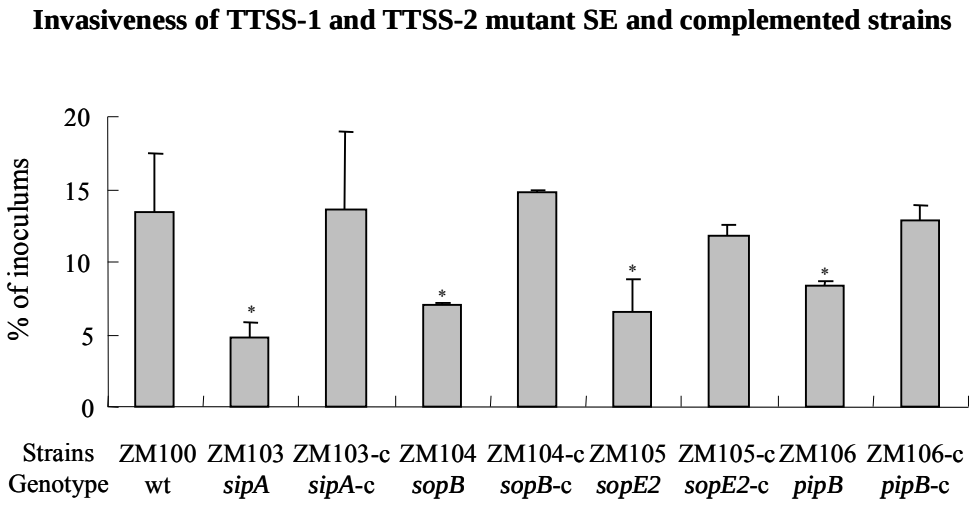

Figure 5 Invasiveness of TTSS-1 and TTSS-2 mutant SE and complemented strains.

Note: Data shown (bars) are geometric means of three independent experiments ± standard deviation. Asterisks indicate that the differences between the invasiveness of mutants and that of the wild type SE are statistically significant ($p < 0.01$). wt, wild type.

3.4. SipA contributes to the intracellular survival of SE inside COEC.

Data from intracellular survival assays showed that mutations in two TTSS-1 effector genes, *sipA* and *sipB*, caused an impaired survival of SE in COEC. Because a

major function of SipB is to translocate effector proteins, including SipA, into the host cell cytosol, the survival defect associated with inactivation of *sipB* was likely due the failure of ZM101 to secrete the SipA protein into COEC. To confirm this notion, plasmid pZM103-c was introduced into mutant ZM103 and the survival rate of the complemented strain was compared with that of ZM100 and ZM103 (Figure 6). Our results indicated that introduction of pZM103-c enhanced the survival of ZM103 to an extent surpassing that of the wild type strain, ZM100.

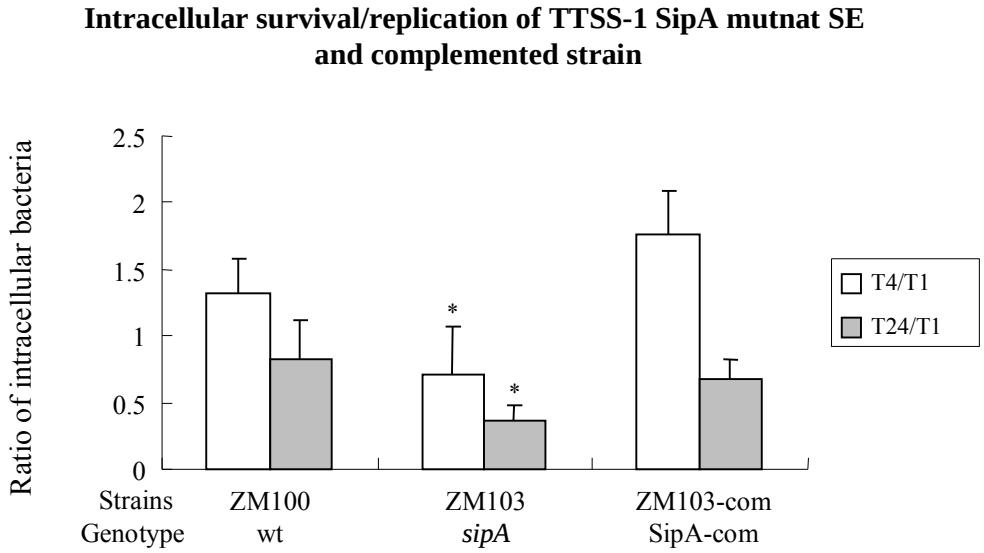

Figure 6 Intracellular survival/replication of TTSS-1 SipA mutant SE and complemented strain.

Note: Net survival/replication of SE strains is presented as ratio between the number of intracellular bacterial at T4 and T1, and T24 and T1. Data shown (bars) are geometric means of three independent experiments ± standard deviation. Asterisks indicate that the net survival of ZM103 was significantly lower than that of the wild type strain ($P < 0.01$). wt, wild type.

3.5. Cytokine and chemokine expression in COEC following infections with SE strains.

In this part of the investigation, real-time RTPCR was conducted to determine SE-induced expressions of several important immune mediators, including pro- or anti-inflammatory cytokines and chemokines, by COEC over a 24-h course of infection. Our results demonstrated that acute pro-inflammatory responses were induced in COEC at 1hpi, peaked at 4hpi, and then declined to minimal levels at 24hpi. Thus, the differences in the amount of cytokine mRNA in COEC cultures infected with different SE strains could only be detected at 4hpi. For example, infection of COEC with ZM100 resulted in an approximately 60-fold increase in iNOS expression which was significantly higher ($P < 0.05$) than that induced by the TTSS mutants. In addition, more iNOS mRNA production in COEC at 4hpi was triggered by TTSS-2 mutants than TTSS-1. From 1hpi to 4hpi, a significant rise in the amount of iNOS occurred in COEC cultures infected with the wild type strain (ZM100) and TTSS-2 mutants (ZM102 and ZM106), whereas only a slight, not statistically significant, up-regulation of iNOS gene was detected in COEC infected with TTSS-1 mutants (ZM101, ZM103, ZM104, and ZM105). A general correlation between the levels of iNOS and the numbers of intracellular bacteria in COEC were observed (Figure 3 and Figure 7).

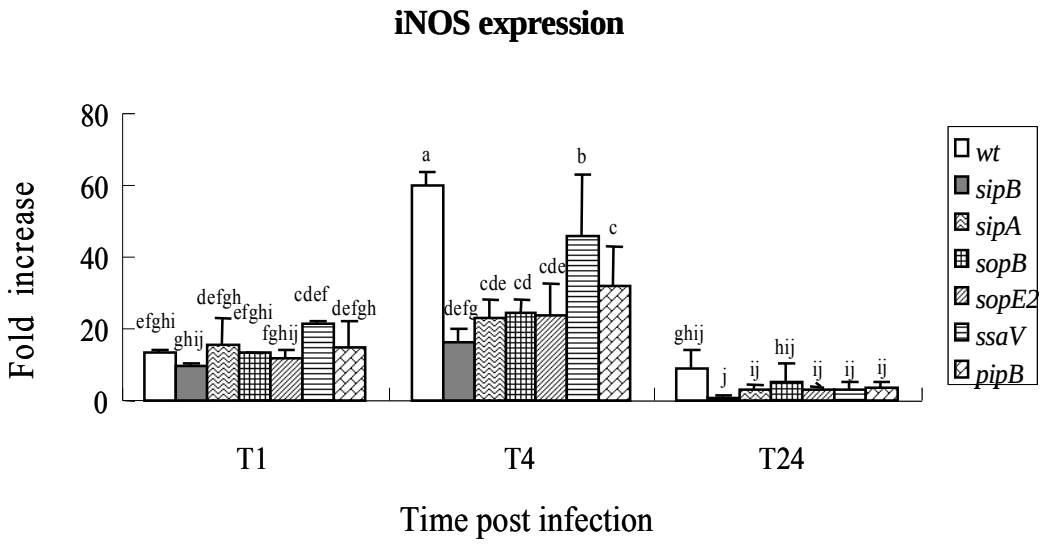

Figure 7 iNOS expression.

Note: The expression levels of iNOS in SE-infected COEC at 1hpi, 4hpi, and 24hpi (T1, T4, and T24) were determined by RT-PCR. Data (bars) are are given as the means from the three independent experiments with standard deviations. Different letters above two bars indicate that the levels of iNOS expressed by COEC cultures are statistically significant different ($P < 0.01$). wt, wild type.

SE infection also induced the synthesis of CXC chemokine (IL-8) and CC chemokine (K203 and MIP 1β). From 1hpi to 4hpi, the amount of IL-8 mRNA increased significantly in COEC cultures infected with ZM100, ZM104 (*sopB*$^-$), ZM102 (*ssaV*$^-$), and ZM106 (*pipB*$^-$), but not ZM101 (*sipB*$^-$), ZM103 (*sipA*$^-$), and ZM105 (*sopE2*$^-$). At the peak expression time (4hpi), mutants ZM101 (*sipB*$^-$) and ZM103 (*sipA*$^-$) induced significantly lower ($P < 0.05$) levels of IL-8 expression than did the wild type strain or other TTSS mutants (Figure 8). SE infection resulted in about 6-fold increase in the transcription of K203. No difference in the expressions of chemokine K203 was found between COEC cultures infected with ZM100 and most TTSS mutants. One exception was that infection with ZM101 (*sipB*$^-$) induced significantly less K203 than other SE strains (Figure 9). SE infection also triggered the transcription of MIP-1β,

another CC chemokine. However, the expression of MIP-1β seemed to be independent of TTSS effectors. In contrast, the *sipA* mutant induced more production of MIP-1β, compared to the wild type strain (Figure 10). In addition, IL-10 was significantly induced by SE infection, which again was not directly associated with individual TTSS effector proteins (Figure 11). The expression of TGF-β3 was suppressed by SE infection through out the course of infection and no significant difference was detected among COEC cultures infected with the wild type and the mutant strains (Figure 12).

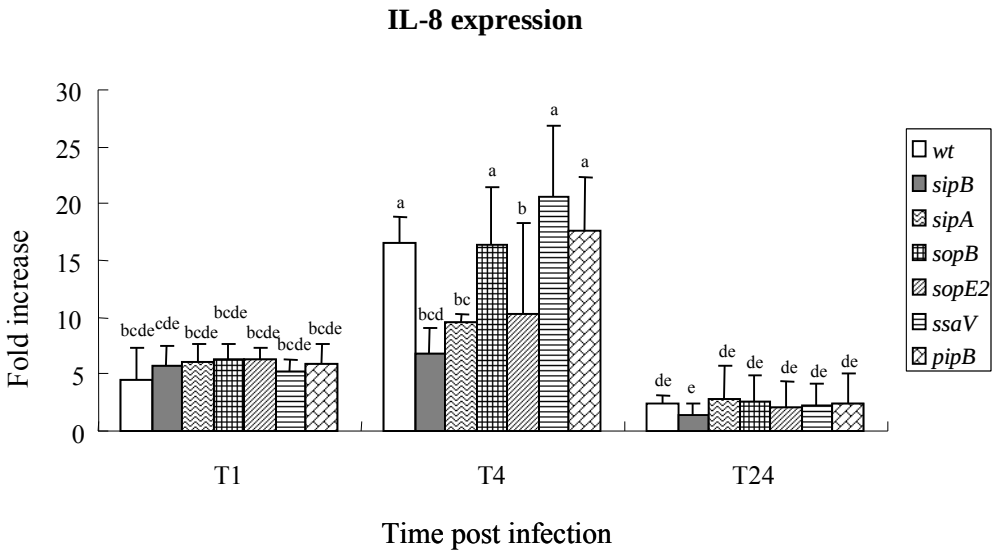

Figure 8 IL-8 expression.

Note: The levels IL-8 expression in SE-infected COEC at T1, T4, and T24 were determined by RT-PCR. The results, presented as fold-increase, are given as the means from the three independent experiments with standard deviations as shown. Different letters above two bars indicate that the results are statistically significant different ($P < 0.01$). wt, wild type.

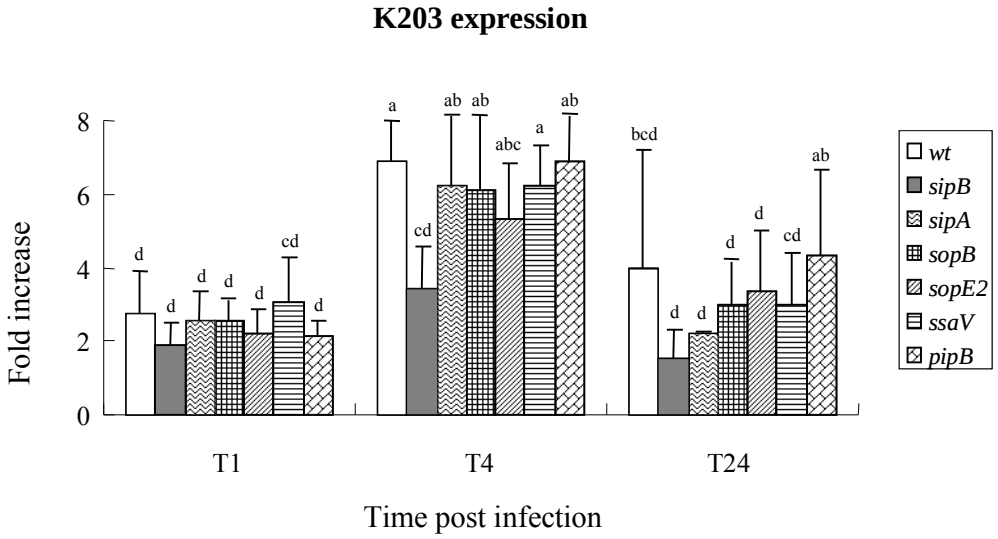

Figure 9 K203 expression.

Note: The levels of K203 in SE-infected COEC at T1, T4, and T24 were determined by RT-PCR. The results, presented as fold-increase, are given as the means from the three independent experiments with standard deviations as shown. Different letters above two bars indicate that the results are statistically significant different ($P < 0.01$). wt, wild type.

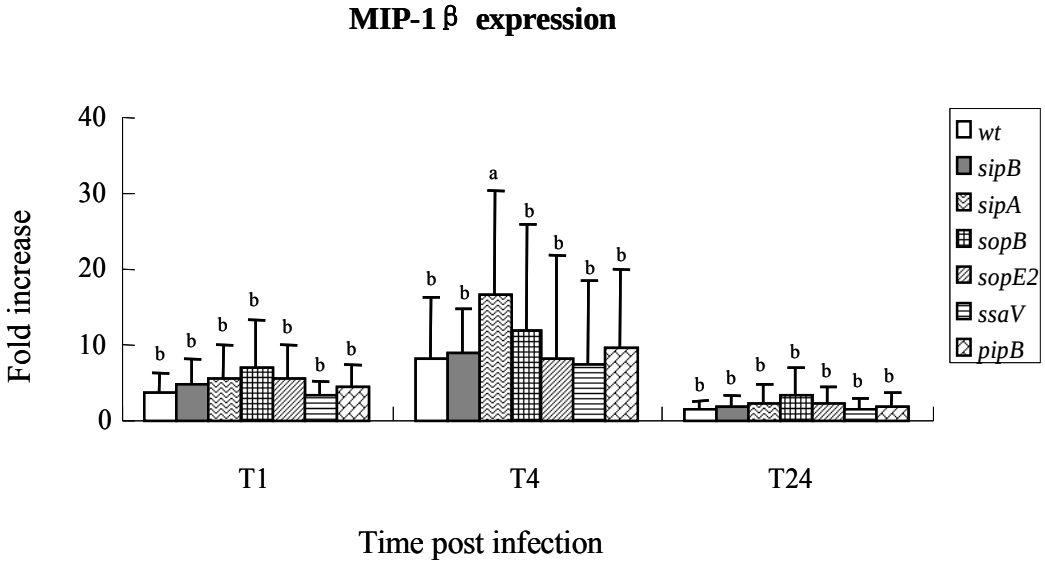

Figure 10 MIP-1βexpression.

Note: The levels of MIP-1β in SE-infected COEC were detected by RT real-time PCR. The results, presented as fold-increase, are given as the means from the three independent experiments with

standard deviations as shown. Different letters above two bars indicate that the results are statistically significant different. wt, wild type.

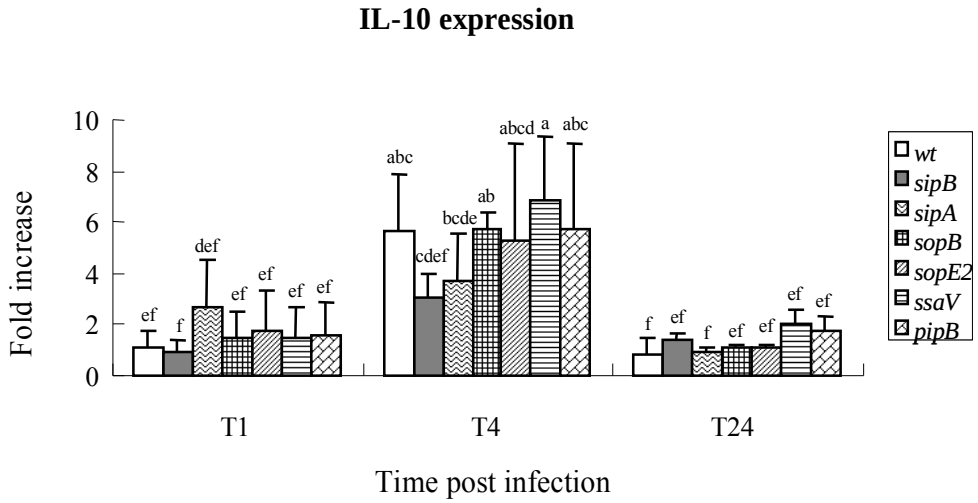

Figure 11 IL-10 expression.

Note: The levels of IL-10 in SE-infected COEC at T1, T4, and T24 were detected by RT real-time PCR. The results, presented as fold-increase, are given as the means from the three independent experiments with standard deviations as shown. Different letters above two bars indicate that the results are statistically significant different ($P < 0.01$). wt, wild type.

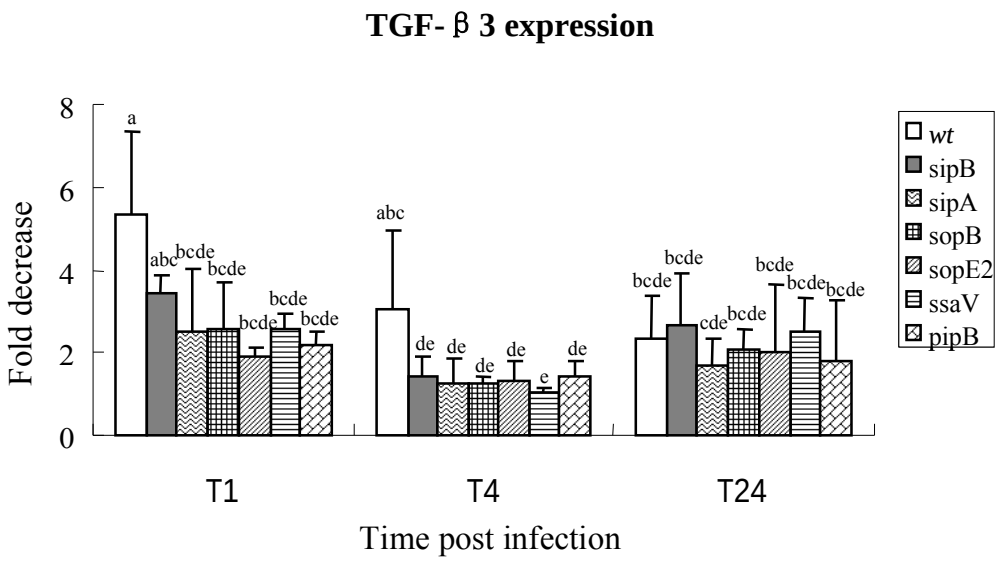

Figure 12 TGF-β3 expression.

Note: The levels of TGF-β3 mRNA expression in COEC at T1, T4, and T24 were detected by RT real-time PCR. The results, presented as fold-decrease, are given as the means from the three independent experiments with standard deviations as shown. Different letters above two bars indicate that the results are statistically significant different ($P < 0.05$). wt, wild type.

SECTION Ⅳ

DISCUSSION

4.1. The pathogenic roles of TTSS-1 and TTSS-2 in SE infection of COEC.

Colonization of the chicken reproductive system by SE is a contributing factor for human SE outbreaks associated with the consumption of contaminated poultry eggs and meats. Although the two virulence associated type three secretion systems (TTSSs) of *Salmonella enterica* have been well characterized using serovar Typhimurium and mammalian infection models, the complex host-pathogen interaction between SE and chicken reproductive system is poorly understood. In an effort to understand the molecular and immunologic mechanisms involved in SE persistent infection of chicken reproductive tract, we assessed the contributions of TTSS-1 and TTSS-2 to SE invasion and survival in primary chicken oviduct epithelial cells as well as their abilities to induce the expressions of immune mediators in infected cells. The epithelial cells used in this study were isolated from the isthmus region of the upper oviduct because it has been identified as a predominant colonization and adhesion site of SE [13, 67]. In addition to a TTSS-1 null mutant, ZM101 (*sipB*$^-$), and a TTSS-2 null mutant, ZM102 (*ssaV*$^-$), four mutants carrying mutations in genes encoding effector proteins of TTSSs, including *sipA*, *sopB*, *sopE*2,

and *pipB* were created, because these genes were necessary for other serovars of *Salmonella enterica* to colonize host intestine or to induce innate immune responses at the site of infection [20, 56, 57]. Consistent with the findings from experiments involving mammalian cells or hosts, we found that ZM101 (*sipB*$^-$), ZM104 (*sopB*$^-$), and ZM105 (*sopE2*$^-$) had significant invasion defects. It has been previously demonstrated that SopB and SopE2 activate Cdc42 signaling, leading to actin cytoskeleton rearrangement macropinocytosis, and bacterial entry [39, 42, 68]. Since the phenotypes of ZM104 and ZM105 in this study are essentially same as that of the *sopB* and *sopE*2 mutant Typhimurium strains in mammalian infection models, it is reasonable to assume that SopB and SopE2 promote SE invasion of COEC via activating Cdc42. SipA is known for its involvement in the very early stage of serovar Typhimurium invasion into mammalian epithelial cells by lowering the concentration of G-actin required for polymerization and stabilizes F-actin at the site of bacterial entry [24, 25]. The *sipA* mutants are as invasive to mammalian epithelial cells as their wild type parent strains when bacteria are recovered at or after 15 minutes post inoculation [24, 25]. Our data showed that ZM103 (*sipA*$^-$) was significantly less invasive to COEC at 1hpi which might suggest a host species-specific role of this gene or its protein product. At the present time, the exact cellular events involved in SipA-mediated SE invasion of chicken cells are unknown. Whether SipA has a different affinity to chicken actin or targets different cellular pathways in chicken epithelia cells remain to be investigated. Nonetheless, data from this study revealed that the three effector proteins, SipA, SopB, and SopE2, are equally important in the

entry of SE into chicken oviduct epithelial cells.

The TTSS-2 is known for its essential roles in the intracellular survival of *Salmonella* in host macrophages and the establishment of systemic infections [69]. An early study suggested an interaction between TTSS-1 and TTSS-2 based on the observation that inactivation of TTSS-2 system reduced the invasiveness of serovar Typhimurium which was correlated with the lack of a TTSS-1 translocase, SipC, in culture supernatant [47]. Our results showed that the two TTSS-2 mutants, ZM102 (*ssaV*⁻) and ZM106 (*pipB*⁻), were less invasive than the wild type strain, ZM100. While the phenotype of ZM102 confirmed the data from an early study on serovar Typhimurium, the role of PipB in SE invasion of COEC differed from all previous findings suggesting this protein was not involved in serovar Typhimurium invasion of mammalian cells lines [20].

In this study, we also identified a role for two TTSS-1genes, *sipA* and *sipB*, in the intracellular survival of SE in COEC. Because a major function of SipB is to translocate effector proteins of TTSS-1 into host cell cytosol, the impaired survival of strain ZM101 could be a result of its inability to translocate SipA into COEC. However, there is no experimental evidence to exclude the possibility that SipB functions as an effector to enhance the survival or replication of SE in COEC. To confirm the role of SipA in SE survival inside COEC, we complemented strain ZM103 with a plasmid pZM103-c encoding the full length *sipA* gene. The results showed that introduction of pZM103-c into ZM103 lead to an enhanced survival rate, compared to ZM103 and even the wild type strain ZM100. It has been recently shown

that SipA, SopB and SopE2 are synthesized at the late stage of the infection in mice, suggesting implication of TTSS-1 in the development of systemic disease [44]. In cultured mammalian epithelial cells, SipA is exposed on the cytoplasmic face of SCV, from where it promotes the replication of serovar Typhimurium. Whether the same mechanism is utilized by SE to survive or replicate in chicken oviduct epithelial cells needs to be verified. Similar to the *sipA* mutant, ZM104 (*sopB*$^-$) showed a reduced survival rate at 4hpi. However, the difference between ZM104 and ZM100 was not statistically significant due to variations among individual experiments. We did not find a significant role for *sopE*2 in the survival of SE in COEC, although this gene was previously implicated in serovar Typhimurium colonization of chick intestine.

It has been demonstrated that inactivation of TTSS-2 renders *Salmonella* unable to replicate in macrophages [52]. In this study, inactivation of *ssaV* and *pipB* conferred SE a replication advantage inside COEC. It has been previously demonstrated that serovar Typhimurium replicates more efficiently in epithelial cell cytosol than inside SCV and the maintenance of SCV membrane was mediated by SifA, an effector protein translocated by TTSS-2 [70]. Thus, the enhanced growth of the *ssaV* mutant in COEC was likely due to the migration of SE organisms from the damaged SCVs to COEC cytosol, an environment permissive to bacterial replication. However, this notion does not explain the increased net replication of ZM106 (*pipB*$^-$) in COEC, unless the PipB protein is also required for the integrity of SCV in chicken cells. Although a pathogenic role could not be assert to PipB in mammalian experimental models, this protein was host-specific colonization factor of

Typhimurium chick intestine [53]. The involvement of PipB in SE invasion of chicken cells observed in this study may provides an explanation for PipB-dependent colonization of chick intestine by serovar Typhimurium, assuming its function is conserved in both serovars in poultry.

4.2. Salmonella enteritidis infection induced expression of immune mediators.

Attempts have been made to understand the host immune responses in the intestine, systemic tissues, and macrophage cultures during *Salmonella* infection of chickens [58, 59, 61]. However, little is known about the innate responses, such as cytokine and chemokine expressions, in chicken reproductive tract colonized by SE. In this study, we determined the levels of several important immune mediators, including iNOS, CXC chemokine IL-8, CC chemokine K203 and MIP-1β, IL-10, and TGFβ-3, in COEC following infections with wild type and TTSS mutant SE strains. Our results showed that the TTSS-1 and TTSS-2 mutants were impaired in iNOS induction, compared to the wild type SE. More interestingly, a significant rise in the amount iNOS mRNA from 1hpi to 4hpi was detected in COEC cultures infected with wild type SE or TTSS-2 mutants, but not TTSS-1 mutants. Based on the expression kinetics, it is reasonable to assume that the TTSS-1 effectors, including SipA, SopB, and SopE, were inducers of iNOS transcription in COEC. However, because a general correlation was found between the numbers of intracellular bacteria and the levels of iNOS in infected COEC, we can not exclude the possibility that the impaired iNOS induction was secondary to the invasion defects of these strains. An early study

indicated that SopE2 was involved in induction of iNOS in mammalian macrophages infected with serovar Typhimurium [28]. To confirm the roles of TTSS-1 effectors in triggering iNOS production, further studies should be carried out to determine the iNOS levels in COEC cultures containing the same numbers of intracellular bacteria, wild type or TTSS-1 mutants, which may be achieved by using a lower dose of wild type SE as inoculum. Nonetheless, iNOS is an important enzyme involved in the production of reactive nitrogen intermediates (RNI) and is required for bacterial clearance [71, 72]. Expression of iNOS by COEC following SE infections suggests that the chicken oviduct epithelium is able to generate oxidative responses against SE which may or may not be potent enough to completely clear the infection.

With other pro-inflammatory immune mediators, IL-8 is a CXC chemokine which functions as a chemoattractant for polymorphonuclear leukocytes (PMN) to the infection site [73] . The effector proteins of TTSS-1, such as SopE2, have been identified as inducers of IL-8 expression in mammalian epithelium [22]. SipA alone can cause PMN transepithlial migration, suggesting a critical role of this protein in induction of inflammatory response in mammalian hosts [46]. In this study, ZM101 (*sipB*$^-$), ZM103 (*sipA*$^-$), and ZM105 (*sopE2*$^-$) induced significantly lower levels of IL-8 expression in COEC, than did the wild type strain and other TTSS mutants. The decreased transcription of IL-8 in COEC infected with ZM101 may be associated with the mutant's severe invasion/survival defects or due to its inability to secrete effector proteins of TTSS-1 into COEC cytosol. However, the impaired induction of IL-8 by ZM103 (*sipA*$^-$) and ZM105 (*sopE2*$^-$) could not be solely attributed to their decreased

invasiveness because similar numbers of intracellular bacteria were recovered from COEC infected with these strains and another TTSS-1 mutant, ZM104 (*sopB*$^-$), and yet, a significantly higher level of IL-8 expression was detected in ZM104-infected cells. These data collectively indicate that the two TTSS-1 effectors, SipA and SopE2, induce IL-8 expression in SE-infected chicken reproductive epithelium.

The CC chemokines, including K203 and MIP-1β, are responsible for the recruitment of monocytes into the site of infection [74]. Our result suggested that SE infection resulted in the expression of both K203 and MIP-1β in COEC, although the magnitude of induction for K203 was not as great as what was observed for MIP-1β or other pro-inflammatory immune mediators. No significant difference in the levels of K203 expression was detected between infections with wild type SE and TTSS mutants, except ZM101 (*sipB*$^-$), suggesting that the TTSS effectors were not involved in the production of K203 by infected COEC. The low level expression of K203 in response to ZM101 (*sipB*$^-$) infection could be associated with the low number of intracellular ZM101, a strain with severe invasion and survival defects. However, our data does not exclude the possibility that SipB has a direct biological function in modulating host transcription. Similar to K203, the expression of MIP-1β was not TTSS-dependent. Interestingly, the *sipA* mutant, ZM103, displayed a small, but significant, advantage in induction of this chemokine.

In mammalian system, IL-10 is responsible for the production of Th2-type of immune response. The chicken IL-10 gene was first cloned in 2004 [75]. The function of IL-10 in chicken is not as clear as in mammals, and it is up-regulated in the

intestinal and systemic tissues following infections with SE or coccidia [75]. Similarly, our data demonstrated that IL-10 gene was up-regulated in chicken oviduct epithelial cells by SE infection. The induction of this cytokine was not associated with any particular TTSS effectors examined. Previously, investigation has demonstrated that TGF- inhibits the production of proinflammatory cytokines via cross talk between MAPKs [76]. TGF-β is an important regulator of inflammation, being pro-inflammatory at low concentrations and the anti-inflammatory at high concentrations [77]. Our data showed that the expression of TGF-β3, an isoform of TGF-β, was down-regulated immediately after inoculation with SE and throughout the course of infection which might further contribute to the enhanced transcription of CXC and CC chemokines. Thus, it seems that chicken oviduct epithelium controls the infection by coordinating the expression of various immune mediators. However, despite the pro-inflammatory responses generated by COEC, a significant proportion of the SE organisms remained alive inside COEC.

A previous investigation demonstrated that TTSS-1 effectors of serovar Typhimurium elicited host-specific chemokine response in animal models of typhoid fever and enterocolitis[78]. The different disease outcomes, namely mild and localized necrosis of Peyer's patches with no diarrhea in mice versus extensive necrosis of ileal mucosa and diarrhea in calves, resulted from the differences in TTSS-1-dependent CXC production by host cells. The results from this study resembled what was found in the enterocolitis model as evidenced by the TTSS-1-induced expression of CXC chemokine, IL-8, but not CC chemokines, K203

and MIP-1β. Unlike Salmonella-induced enterocolitis that is accompanied with extensive tissue damage, gross or histopathological changes rarely occur in the reproductive system of laying hens naturally infected with SE. Whether this is due to a suboptimal potency or duration of the pro-inflammatory responses needs to be further investigated using in vivo infection models.

Taken together, data from the present study have provided important insights into the complex pathogen-host interactions during SE infection of chicken oviduct epithelial cells. This study has not only confirmed several important roles of TTSS effectors in *Salmonella* invasion of host cells and induction of host immune responses, but also revealed a number of serovar- and host species-specific functions for TTSS-1/2 effectors. The unique findings from our bacterial invasion and survival assays can be summarized as the following: 1) both TTSS-1 and TTSS-2 contribute to SE invasion of COEC, 2) SipA is as important as SopB and SopE2 in the entry of SE into COEC, 2) SipA is also involved in the survival of SE inside COEC, and 3) PipB is required for invasion and possibly the integrity of SCV. In addition, this study has, for the first time to the author's knowledge, assessed the innate immune responses elicited by SE and its TTSS effectors. The results of cytokine expression analyses can be outlined as 1) SE infection triggers pro-inflammatory responses in COEC, 2) TTSS-1 effectors, SipA, SopB, and SopE2 are involved in the expression of iNOS, 3) SipA and SopE2 are inducers of CXC chemokine, IL-8, 4) TTSS-1/2 effectors are not necessary for eliciting CC chemokine K203 and MIP-1β, or cytokine IL-10, and 5) TTSS-1/2 effectors are not involved in the suppression of TGF- β3 in COEC.

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APPENDIX A

BUFFERS AND MEDIA

1. Buffer for Southern Hybridization analysis

1.1 Denature Buffer for Southern Hybridization analysis

Sodium hydroxide (0.5 N)

Sodium chloride (1.5 M)

1.2 Neutralization buffer for Southern Hybridization analysis

Tris pH7.5 (1 M)

Sodium chloride (1.5 M)

1.3 Hybridization buffer

Hybridization buffer (AlkPhos Direct Labelling Kit)

Sodium chloride (0.5M)

Blocking reagent (AlkPhos Direct Labelling Kit, 4% (w/v))

1.4 Primary wash buffer for Sounthern Hybridization analysis

Urea (2M)

SDS (0.1% (w/v))

Sodium Phosphate (50mM, pH 7.0)

Sodium chloride (150mM)

Magnesium chloride (1mM)

Blocking Reagent (AlkPhos Direct Labelling Kit, 0.2% (w/v))

1.5 Secondary wash buffer for Sounthern Hybridization analysis (20* stock)

Tris base (1M)

Sodium chloride (2M)

1.6 Secondary wash buffer working solution

20* stock secondary wash buffer

1M Magnesium chloride (2 mM)

DI water

2. Media for chicken oviduct epithelial cells

2.1 Wash buffer for chicken oviduct epithelial cells.

Hanks' Balanced Salt Solution (Sigma)

Gentamicin (Sigma, final concentration 0.1mg ml^{-1})

Penicillin-Streptomycin Solution (Sigma, final concentration 200 units and 0.2mg ml^{-1} respectively)

Amphotericin B (Sigma, 5μg ml-1)

2.2 Collagenase treatment for chicken oviduct epithelial cells

PBS (Calcium and Magnesium free, Sigma)

Collagenase (from Clostridium histolyticum, type Ⅺ, Sigma, 1mg ml^{-1} final)

Gentamicin (Sigma, 100μg ml^{-1} final)

Penicillin/Streptomycin Solution (Sigma, 400 units and 0.4mg ml^{-1} final, respectively)

2.3 Chicken oviduct epithelial cells culture media

Minimal Essential Media Eagle (Sigma)

Fetal bovine serum (Gibco Invitrogen, 15% final)

Insulin (Sigma, 0.01mg ml^{-1} final)

Estrogen (Sigma, 0.05mM final)

Penicillin-streptomycin (Sigma, 100 unites and 0.1mg ml^{-1} final, respectively)

Gentamicin (Sigma, 25μg ml^{-1} final)

2.4 Invasion media for chicken oviduct epithelial cells

Minimal Essential Media Eagle (Gibco Invitrogen)

Insulin (Sigma, 0.01mg ml^{-1} final)

Estrogen (Sigma, 0.05mM final)

2.5 Gentamicin treatment media for chicken oviduct epithelial cells

Minimum Essential Medium Eagle (Sigma)

Fetal Bovine Serum (Gibco Invitrogen, 5% final)

Insulin (Sigma, 0.01mg ml^{-1} final)

Estrogen (Sigma, 0.05mM final)

Gentamicin (Sigma, 50μg ml^{-1} final)

2.6 Maintenance media for chicken oviduct epithelial cells

Minimum Essential Medium Eagle (Sigma)

Fetal Bovine Serum (Gibco Invitrogen, 5% final)

Insulin (Sigma, 0.01mg ml^{-1} final)

Estrogen (Sigma, 0.05mM final)

Gentamicin (Sigma, 25μg ml^{-1} final)

3. Media for electro-complement cells

No Salt Luria Bertani (LB)

BBL™ Yeast Extract	5g L^{-1}
Bacto™ Tryptone	10g L^{-1}

APPENDIX B

PROCEDURES

1. T-A TOPO cloning (Invitrogen) and chemical transformation:

1.1 Gently mix 4μl fresh PCR product, 1μl Salt solution from the kit and 1μl pCR2.1 TOPO Vector together.

1.2 The mixture was sit at room temperature for 15 minutes.

1.3 The mixture was transferred to a vial of One Shot® E.coli, which was thawed on ice, with gently mixing.

1.4 Incubate on ice for 30 min.

1.5 The heat-shock was performed after incubation on ice at 42℃ for 30 seconds without shaking.

1.6 The tube was immediately transferred to ice and 300μl of room temperature S.O.C. medium, followed by 2 hours' shaking.

1.7 100μl culture was spread on a prewarmed LB plate with ampicillin 100μg ml^{-1} and X-gal 30μg ml^{-1}.

1.8 The bacterial culture plates were incubated at 37℃ for at least 12 hours.

1.9 Pick up the white colonies and subculture on new plates.

1.10 Confirm insertion by using PCR-screening.

2. Plasmid extraction by using Wizard® Plus Minipreps DNA Purification System from Promega

2.1 Fully resuspend a full loop (10μl inoculation loop) of bacterial containing plasmids in 0.3ml Cell Resuspension Solution, leaving no bacterial clumps.

2.2 Add 0.3ml of Cell Lysis Solution, mix gently by inverting the tube 4-6 times, and incubate at room temperature for 5 minutes.

2.3 Add 0.3ml of chilled Neutralization Solution, mix immediately but gently by inverting the tube 4-6 times, and incubate on ice for 5 minutes.

2.4 Centrifuge the bacterial lysate at 13,200rpm for 10 minutes.

2.5 Assemble the Wizard® Minicolumns on the Vac-Man® (20-sample capacity) Laboratory Vacuum Manifold connected to the Vacuum.

2.6 Apply 1ml Column Wash Solution in each Wizard® Minicolumns

2.7 Transfer the cleared lysate to the prepared Wizard® Minicolumn. Start the Vacuum.

2.8 When all liquid has been pulled through the column, apply 1ml Wizard® Minipreps DNA Purification Resin previously diluted with 95% ethanol in the Wizard® Minicolumn, twice.

2.9 When all liquid has been pulled through the column, release the vacuum.

2.10 Spin the column on the top of empty collection tube at 13,200rpm for 1 mintue.

2.11 Remove the Wizard® Minicolumins new collection tubes. Elute the DNA from the Wizard® Minicolumn in Nuclease-Free Water. Sit in room temperature for 2 minutes.

2.12 Collect the DNA by centrifugation at 13,200rpm for 1 minute.

2.13 The DNA can be used immediately or saved in -20℃ for long term storage.

3. Conjugation

3.1 Culture *E.coli* and SE strains overnight

3.2 Subculture 100µl overnight culture in new TSB broth, with 4 hours' shaking.

3.3 Transfer 1ml 4 hours' *E. coli* culture in 1.5ml centrifuge tube, centrifuge at 13,200rpm for 5 minutes. Remove the supernatant.

3.4 Add 1ml 4 hours' SE culture in the tube. Centrifuge at 13,200rpm for 5 minutes. Remove the supernatant. Only leave 60µl in the tube.

3.5 Resuspend bacterial pellet in 60µl media.

3.6 Place the bacterial drop on LB plate without antibiotics. Air dry. And then incubate the plate at 37℃ overnight.

4. Recover the DNA from agarose gel by using Wizard® SV Gel and PCR Clean-Up System

4.1 The digestion products were separated by running 0.8% agarose gel and the insertion band was cutted from the gel.

4.2 The gel slices were weighted and the same amounts of membrane binding solution were added into the tubes.

4.3 The mixture were incubated in 56℃ till the gel slices dissolved.

4.4 Dissolved gel mixtures were transferred to the SV Minicolumn assembly and incubated for 1 minute at room temperature, followed by centrifugation at 13,200rpm for 1 min.

4.5 Remove the SV Minicolumn from the Spin Column assembly and discard the liquid in the Collection Tube, and then return the SV Minicolumn back to the Collection Tube.

4.6 Wash the column by adding 700µl of Membrane Wash Solution, which was previously diluted with 95% ethanol to the SV Minicolumn, followed by 5 minutes' incubation at room temperature and centrifugation for 1 minute at 13,200rpm. Discard the liquid in the collection tube.

4.7 Wash the SV Minicolumn again with 500µl of Membrane Wash Solution and followed by centrifugation for 5 minutes at 13,200rpm.

4.8 Replace the Collection Tube by a new one from the assembly.

4.9 Spin the assembly for 1 minute at 13,200rpm. And then carefully remove the SV Minicolumn from the assembly to a new 1.5ml centrifuge tube.

4.10 Apply 20μl of DNase RNase free water to the center of the SV Minicolumn without touching the membrane. Incubate at room temperature for 2 minutes and centrifuge for 1.5 minute at 13,200rpm.

4.11 Discard the SV Minicolumn and store the centrifuge rube containing the eluted DNA at -20℃.

5. T4 Ligation by using the kit from Invitrogen

5.1 Reaction system (all the buffers and enzymes are from T4 Ligation Kit)

Recovered DNA	1μl,
Double digested pEP185.2	2μl,
5* Buffer	2μl,
DNA ligase	1μ,
DNase-RNase-free water	4μl,

5.2 Incubated at room temperature for 20 minutes.

6. Making competent cell S17λ-*pir*

6.1 warm up 1 tube of TSB to 37℃.

6.2 Suspend a heavy loop of S17λ-*pir* in TSB with 200rpm shaking for 30 minutes.

6.3 Take 200μl culture to 200ml LB (pre-warmed) with 20rpm shaking for 4 hours, till the

OD600 to 0.4.

6.4 Immediately chill the culture on ice, separate the 200ml culture into four 50ml tubes and keep on ice for 15 minutes.

6.5 Harvest the bacterial by centrifugation at 2000rpm for 10 minutes at 4℃. Remove the supernatant.

6.6 Resuspend the bacterial pellet in 20ml ice-cold 0.1M Calcium Chloride. Incubate on ice for 20 minutes.

6.7 Harvest bacterial by centrifugation at 2000rpm for 10 minutes at 4℃. Remove the supernatant.

6.8 Resuspend the bacterial pellet in 10ml ice-cold 0.1M Calcium Chloride.

6.9 Combine four tubes of bacterial together to one tube.

6.10 Harvest bacterial by centrifugation at 2000rpm for 10 minutes at 4℃. Remove the supernatant.

6.11 Resuspend the bacterial pellet in 5ml ice-cold 0.1M Calcium Chloride.

6.12 Incubate on ice overnight.

6.13 Add 1.2ml sterile glycerol. Mix thoroughly.

6.14 Separate bacterial suspension in 1.5ml sterile centrifuge tubes.

6.15 Store at -80℃.

7. Genomic DNA extraction by using CTAB (hexadecyltrimethylammonium bromide)

7.1 Fully resuspend a full sterile inoculation loop (10μl) of bacterial in 500μl TE buffer with 100μl 10% of SDS and 5μl Proteinase K (30mg ml^{-1}). Incubate in 65℃ water bath for 1

hour.

7.2 Add 100µl 5M sodium chloride and 80µl 10% CTAB in 0.7 M sodium chloride. Incubate in 65℃ water bath for 20 minutes.

7.3 Add 500µl chloroform in tube, and mix by vertex. Centrifuge at 13,200rpm for 10 minutes.

7.4 Transfer the upper layer to a new tube, and add 500µl phenol, mix well by vertex. Centrifuge at 13,200rpm for 10 minutes.

7.5 Transfer the upper layer to a new tube again, and add 500µl phenol-chloroform, well mixed, followed by centrifuge at 13,200rpm for 10 minutes.

7.6 Purify the DNA with 500µl chloroform again, followed by centrifugation at 13,200rpm for 10 minutes.

7.7 Genomic DNA was precipitated by adding 500µl isopropanol and 25µl 3M NaAC.

7.8 Collect the genomic DNA by centrifugation at 13,2000rpm for 30 minutes.

7.9 DNA pellet was washed by 70% ETOH.

7.10 Air dry. Apply 100µl DNase RNase free water to elute the DNA pellet.

8. Genomic DNA electrophoresis

Digested Genomic DNA was loaded into 0.8% Agarose gel, 125V till the front Dye goes to the bottom of the gel.

9. Southern Hybridization analysis

9.1 Gel denature and neutralization

9.1.1 Cut the edge of the gel without DNA and label the DNA marker with DNA loading buffer.

9.1.2 Place the gel in a glass dish and cover the gel with denature buffer. Rotate the gel gently for 30 minutes.

9.1.3 Discard the denature buffer and rinse the gel with DI water.

9.1.4 Add neutralization buffer, and rotate the gel gently for 30 minutes.

9.1.5 Change fresh neutralization buffer, and rotate for another 15 minutes.

9.2 Set up transfer apparatus

9.2.1 Use middle size gel horizontal system as container and the transfer buffer is 10*SSC buffer.

9.2.2 Prepare two pieces of long 3mm Whatman papers, and wet the papers thoroughly with transfer buffer. Apply the papers on the top of the support, one over another, avoiding air bubble.

9.2.3 Place the gel on the top of the Whatman 3mm papers. Avoid air bubble.

9.2.4 Cut Hybond-N+ membrane (Amersham Biosciences) into the same size as the gel, wet thoroughly with transfer buffer and apply on top of the gel. Smooth out air bubble.

9.2.5 Place the plastic wraps around the gel. Do not cover the gel.

9.2.6 Add transfer buffer into container till the level of the liquid reaches almost to the top of the support.

9.2.7 Thoroughly wet a piece of 3mm Whatman paper with the same size as the gel, and then place on the top of the membrane. Avoid air bubble.

9.2.8 About 20 cm thick of paper towels were placed on the top of the 3mm Whatman paper. Put a glass plate on the top of the stack and weight it down with 500g weight.

9.2.9 Use plastic wraps cover the buffer to prevent the buffer evaporation.

9.2.10 This apparatus should be kept for 18 to 24 hours, the paper towels were replaced if necessary.

9.3 Cross link the membrane

9.3.1 Carefully remove the entire stack on the top of the Hybond-N+ membrane. Place the membrane on a piece of paper towel till the membrane is dry.

9.3.2 Label the DNA marker with pencil and also label the direction of the order of the DNA on the membrane.

9.3.3 Covered with another paper towel, crosslink the membrane with the DNA by using XL-1500 UV crosslinker (Spectronics Corporation) twice.

9.3.4 Wrap the membrane with plastic wrap and store at 4℃ till the probe is ready.

9.4 Preparation of labeled probe

9.4.1 PCR amplify the internal fragment for each target gene by using appropriate primers listed in Table2.2. Purify the PCR product with once phenol-chloroform, isopropanol and 3MNaAC, and wash the DNA pellet by using 70% ethanol.

9.4.2 Elute the DNA pellet with 10µl DNase-RNase free water.

9.4.3 The cross-linker working concentration solution was made by mixing 10µl cross-linker solution and 40µl of water supply from AlkPhos Direct Labelling Kit.

9.4.4 The PCR product was diluted to a concentration of 10ng μl^{-1} with water supplied from AlkPhos Direct Labelling Kit, and 10µl of diluted PCR product was placed in a

microcentrifuge tube and denatured by heating at 97℃ in Eppendorf Mastercycler for 5 minutes, then immediately cooled on ice for 5 minutes, followed by briefly spin.

9.4.5 Add 10µl of reaction buffer (AlkPhos Direct Labelling Kit) to cooled DNA with gently mixing thoroughly.

9.4.6 Add 2µl labelling reagent (AlkPhos Direct Labelling Kit), brief spin.

9.4.7 Add 10µl of cross-linker working solution, with gently mixing thoroughly and brief spin.

9.4.8 Incubate the reaction at 37℃ for 30 minutes

9.4.9 The probe can be used immediately or kept on ice for up to 2 hours.

9.5 Pre-hybridization and Hybridization

9.5.1 Preheat the hybridization buffer to 55℃ in Hybridization oven (Fisher Scientific).

9.5.2 Place the membrane in a hybridization tube, wet the membrane with about 15ml prewarmed hybridization buffer.

9.5.3 Pre-hybridize for 1.5 hours in hybridization oven.

9.5.4 Add probes into the hybridization tube.

9.5.5 Hybridization at 55℃ overnight in hybridization oven.

9.6 Post hybridization

9.6.1 Preheat the primary wash buffer to 55℃.

9.6.2 Discard the hybridization buffer in the hybridization tube. Add about 20ml warm primary wash buffer. Put the tube back to the hybridization oven and wash for 10 minutes at 55℃.

9.6.3 Perform a further wash with primary wash buffer at 55℃ for another 10 minutes.

9.6.4 Take out the membrane from the hybridization tube and place in a clean container.

9.6.5 Add secondary wash buffer working dilution in the container with gentle agitation for 5 minutes at room temperature.

9.6.6 Wash again with secondary wash buffer working dilution.

9.6.7 Discard wash buffer, and add CDP-Star™ Detection Reagent by pipette on to the membrane for 5 minutes.

9.6.8 Wrap the membrane with plastic wrap, and smooth out the air bubbles. Capture the picture with Kodak Image Station IS2000MM.

10. Extraction of low-copy-number plasmid by Qiagen Plasmid Midi Kit

10.1 A single confirmed bacterial colony was picked from the LB plate and then cultured in 100ml LB broth with ampicillin 100μg ml-1 with vigorous shaking 300rpm at 37℃ for 14 hours.

10.2 Harvest the cells by centrifugation at 4,000rpm for 40 minutes.

10.3 Discard the supernatant and fully resuspend the bacterial pellet into 4ml P1 buffer (Resuspension Buffer).

10.4 Add 4ml P2 buffer (Lysis Buffer), and mix well by inverting the tube for 4-6 times gently. Sit for 5 minutes at room temperature.

10.5 Add 4ml chilled P3 buffer (Neutralization Buffer), and mix well by inverting for 4-6 times gently. Incubate on ice for 30 minutes.

10.6 The mixture was centrifuged at 4,000rpm for 45 minutes. Transfer the supernatant into a new tube. Centrifuge again at the same speed for 15 minutes. The supernatant was

transferred into a new tube.

10.7 Equilibrate a Qiagen-tip100 by applying 4ml Buffer QBT and empty the column by gravity flow.

10.8 Apply the supernatant from previous step to Qiagen-tip100 and allow the supernatant enter the resin by gravity flow.

10.9 Wash the Qiagen-tip100 with 10ml Buffer QC twice.

10.10 Elute the plasmid with 5ml Buffer QF. Collect the elution and separate into five 2ml centrifuge tubes as 1ml per tube.

10.11 Precipitate the DNA by adding 0.7ml isopropanol and mix by vertex.

10.12 Centrifuge at 13,200rpm for 20 minutes.

10.13 Discard the liquid and wash the pellets with 1ml 70% ETOH. Centrifuge at 13,200rpm for 10 minutes.

10.14 Repeat the washing with 70% ETOH for 3 times. Discard the ETOH.

10.15 Air dry. Elute the plasmid with DNase-RNase free water. Combine 5 tubes of elution to 1 tube to make the total amount of elution as 100μl.

11. Making electro-competent cell

11.1 A single colony was inoculated in 50ml No Salt LB with nalidixic acid 50μg ml^{-1} at 37℃ 200rpm overnight.

11.2 Prewarm two 200ml No Salt LB in 37℃.

11.3 Subculture 5ml overnight culture to each 200ml No Salt LB. Shake culture to OD600=0.6 to 0.75.

11.4 Immediately chill the culture on ice.

11.5 Separate the culture into 50ml centrifuge tube. Harvest cells by centrifugation at 4℃ 4,000rpm for 15 minutes. Carefully remove the supernatant.

11.6 Resuspend bacterial pellet in 20ml ice-cold 10% glycerol. Combine two of the tubes together. Harvest cells by centrifugation at 4℃ 4,000rpm for 15 minutes. Carefully remove the supernatant.

11.7 Repeat step 11.6 twice.

11.8 Resuspend the bacterial pellet in 1ml ice-cold 10% glycerol.

11.9 Separate bacterial suspension in 1.5ml sterile centrifuge tubes as 220μl in each tube.

11.10 Store in -80℃

12. RNA extraction

12.1 Add chloroform into Trizol (Invitrogen) as 1:5(v/v). Mix well by vertex, and sit at room temperature for 2 minutes.

12.2 Centrifuge at 13,200rpm for 15 minutes.

12.3 Transfer 80% upper layer to new tube.

12.4 Add equal volume of isopropanol. Mix well by vertex.

12.5 Sit at -80℃ for 30 minutes.

12.6 Centrifuge at 13,200rpm for 10 minutes. Discard liquid.

12.7 Add 70%ETOH to wash the pellet without touching by tip.

12.8 Centrifuge at 13,200rpm for 5 minutes. Discard ETOH.

12.9 Air dry. Elute RNA with RNase-DNase free water.

13. RNA electrophoresis

RNA was mixed with RNA loading buffer (Sigma) and then loaded into 1.5% agarose gel, 125V till the front Dye goes to the middle of the gel.

14. phenol-chloroform purify DNA

14.1 The volume of the DNA should be over 100μl, if not, add RNase-DNase free water to at least 100μl.

14.2 Add phenol to the DNA solution as 1:2 (v/v). Mix well. Centrifuge at 13,200rpm for 5 minutes. Transfer upper layer to a new tube.

14.3 Add chloroform to DNA solution as 1:2 (v/v). Mix well. Centrifuge at 13,200rpm for 5 minutes. Transfer the upper layer to another new tube.

14.4 Add isopropanol into DNA solution as 1:1 (v/v), and also add Sodium Acetate (2M pH 4.0) as 1:10 (v/v). Sit at -80℃ for 30 minutes. Centrifuge at 13,200rpm for 30 minutes. Discard the liquid.

14.5 Add 70%ETOH without touching the DNA pellet by tip. Centrifuge at 13,200rpm for 5 minutes.

14.6 Air dry. Apply appropriate amount of RNase-DNase free water to elute the DNA.

14.7 Store at -20℃.

15. PCR reaction systems (the buffers and the enzymes are from Sigma)

15.1 Amplify sequences reaction

DNA template	0.2µl
Forward primer (10µM)	0.5µl
Backward primer (10µM)	0.5µl
dNTPs	2µl
10*buffer	5µl
$MgCl_2$	6µl
Taq Polymerase	1µl
DNase free water	34.8µl

15.2 PCR-screening reaction

DNA template	0.1µl
Forward primer (10µM)	0.2µl
Backward primer (10µM)	0.2µl
dNTPs	0.75µl
10*buffer	2µl
$MgCl_2$	2µl
Taq Polymerase	0.3µl
DNase free water	14.5µl

15.3 Program

Based on different primers Tm value, and amplicon length, to set up annealing temperature and elongation time.

16. Reverse transcription reaction (the buffers and the enzymes are from Applied Biosciences)

Mixture 1

10*buffer	7μl
$MgCl_2$	6μl
Inhibitor	0.5μl
DNase Ⅰ	0.5μl
mRNA	21μl

Mixture 1 was sit in room temperature for 15 minutes, and then performed heat inactivation at 75℃ for 10 minutes. Mixture 1 and mixture 2 was mixed together when mixture 1 was cooled down to the room temperature.

Mixture 2

DNase RNase free water	27μl
Inhibitor	0.5μl
dNTPs	3.5μl
Hexamer	0.5μl
Reverse Transcriptase	1.5μl

Program:

25℃	10 minutes
48℃	40 minutes
95℃	10 minutes

17. Real-time PCR reaction (SYBR Green from Applied Biosciences)

Diluted and mixed primers for each cytokine or chemokine:

DNase RNase Free Water	90µl
Forward primer (100µM)	5µl
Backward primer (100µM)	5µl

Real-time PCR:

SYBR Green Mixture	12.5µl
Mixed Primers	2µl
DNase RNase free water	3µl
cDNA	7.5µl

Program:

Stage	Temperature	Time (minute)	Repeat
1	95℃	5:00	1
2	95℃	0:15	50
	55℃	0:15	
	72℃	0:30	
3 (Dissociation)	95℃	0:15	1
	60℃	0:20	
	95℃	0:15	