THE ROLE OF ANTIMICROBIAL PEPTIDE RESISTANCE GENES, *VIRK* AND *YBJX*, DURING *Salmonella enterica* SEROVAR ENTERITIDIS INFECTION IN THE CHICKEN REPRODUCTIVE TRACT

A Thesis

by

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ABSTRACT

Salmonella enterica serovar Enteritidis (*S.* Enteritidis) is a major etiologic agent of non-typhoid salmonellosis, which causes 1.028 million cases with approximately 400 deaths in the United States. *S.* Enteritidis persistently and silently colonizes the intestinal and reproductive tract of laying hens, resulting in contaminated poultry products. The consumption of contaminated poultry products has been identified as a significant risk factor for human salmonellosis. To understand the mechanisms *S.* Enteritidis utilizes to colonize and persist in laying hens, we used selective capture of transcribed sequences to identify genes over-expressed in the chicken macrophage cell line (HD11) and in primary chicken oviduct epithelial cells. From the 15 genes found to be overexpressed in both cell types, we characterized the antimicrobial peptide resistance genes (AMPR), *virK* and *ybjX*, *in vitro* and *in vivo*. *In vitro,* AMPR genes were required for natural morphology, motility, secretion, defense against detergents such as EDTA and bile salts, and resistance to antimicrobial peptides polymyxin B and avian β-defensins. From this, we inferred the AMPR genes play a role in outer membrane stability and/or modulation. AMPR genes also played distinct roles in macrophage invasion and survival. In laying hens, both AMPR genes were involved in early intestinal colonization and fecal shedding. In the reproductive tract, *virK* was required in early colonization while a deletion of *ybjX* caused increased ovary colonization and egg deposition. In conclusion, data from the present study indicate that AMPR genes are differentially utilized in various host environments to defend against host immunity, with the possibility this is

through mechanisms that modulate the outer membrane; this ultimately assists *S.* Enteritidis in persistent and silent hen colonization. Decoding the specific mechanisms employed by *S.* Enteritidis during colonization will aid in better control mechanisms to reduce this pathogen's prevalence.

DEDICATION

I dedicate this work to my loving husband. Although he may not understand this work, he has all been very supportive during my pursuit of this degree. I also appreciate that he always wants me to accomplish my very best in whatever I pursue.

I also want to dedicate this work to my P.I. Dr. Shuping Zhang, if it was not for her persuasiveness, positive attitude, persistence, and belief in me, I could not have finished this work. Her support, character, and ability to handle obstacles has made me strive to be more like her; which is how most of this research was accomplished.

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NOMENCLATURE

S. Enteritidis	*Salmonella enterica* subsp. *enterica* serovar Enteritidis
S. Typhimurium	*Salmonella enterica* subsp. *enterica* serovar Typhimurium
AMPR	Antimicrobial peptide resistance
HD11	Chicken macrophage cell line
COEC	Primary chicken oviduct epithelial cells
AvBD	Avian β-defensins
SCOTS	Selective capture of transcribed gene sequences
T3SS	Type three secretion system
SPI	*Salmonella* pathogenicity island
SCV	*Salmonella* containing vacuole
h	Hours
hpi	Hours post infection
dpi	Days post infection
WT	Wild type *S.* Enteritidis strain ZM100
virK	"Insertion mutant" with gene *virK* inactivated
ybjX	"Insertion mutant" with gene *ybjX* inactivated
Δ*virK*	"Deletion mutant" with gene *virK* deleted
Δ*ybjX*	"Deletion mutant" with gene *ybjX* deleted
Δ*virK*Δ*ybjX*	"Deletion mutant" with genes *virK* and *ybjX* deleted

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1. INTRODUCTION

Salmonella enterica subsp. *enterica* (*Salmonella*) serovars are important zoonotic pathogens that cause 1.028 million non-typhoidal salmonellosis cases with approximately 400 deaths annually in the United States. *Salmonella* serovars were also the leading cause in hospitalizations and deaths in the United States from 2000 to 2008 when compared to other food-borne pathogens [1]. Since 1994, *Salmonella* serovar Enteritidis (*S.* Enteritidis) has been the predominate serovar isolated from non-typhoidal salmonellosis cases in the United States [2]. A majority of these cases are associated with consumption of poultry meat or egg products. Since the 1990s, eggs have been a significant source of infection and chicken products have been identified as a significant risk factor for illness [2-4]. In the 1990s, the USDA and FDA implemented regulations on quality control, storage, and transportation, and led efforts to improve consumer knowledge of proper storage and cooking of eggs; this resulted in a 50% decrease in *S.* Enteritidis induced illnesses by 1999 [4]. Even with increased surveillance and regulations, *S.* Enteritidis infections continue to be a major health concern that leads to economic losses. For example, in August of 2010 the United States had to recall 500 million eggs during an outbreak of *S.* Enteritidis in Iowa that spread to 11 states and caused over 1,939 illnesses [5].

Egg contamination can be a result of horizontal transmission, from the environment after being laid, or vertically being contaminated from *S.* Enteritidis colonizing the reproductive tract [2]. Chicken farm houses serve as a reservoir for

Salmonella infection in chickens via the fecal-oral route as *Salmonella* is horizontally transmitted by contaminated fecal material and from the environment. In the environment, *Salmonella* has been isolated from insects, rodents, and birds near farm houses [2, 6]. After ingestion, the mild acidic crop primes *Salmonella* for the acidic environment faced in the intestinal tract, where *Salmonella* withstands this pressure due to up-regulation of stress response genes such as the RpoS regulon [7]. The *rpoS* gene encodes sigma factor 38 that is induced during stationary phase or during stress responses [8, 9]. *Salmonella* utilizes another stress response regulon controlled by PhoP/PhoQ, a regulon that is speculated to modulate the outer membrane of *Salmonella* to contribute to its survival in acidic and low Mg^{+2}/Ca^{+2} conditions [10]. In *Salmonella* serovar Typhimurium (*S.* Typhimurium), it was shown that loci in the PhoP/PhoQ regulon were required for resistance to bile acid and antimicrobial peptides [11-13]. Using these stress responses, *Salmonella* is able to evade innate immune responses in the intestinal lumen, such as bile salts and avian β-defensins (AvBDs), to interact with the epithelium [13, 14]. It has also been shown that not only do these stress responses help *Salmonella* survive in host environments, but genes in these regulons participate in virulence, such as invasion into the intestinal tract and persistence [7, 11, 15, 16].

In the intestine, after survival in the lumen conditions, *Salmonella* is either translocated into the lamina propria by M cells or lumen-sampling dendritic cells, or *Salmonella* invades intestinal epithelial cells to initiate infection [14]. *Salmonella* species contain two known pathogenicity islands, *Salmonella* pathogenicity island 1 and 2 (SPI 1 & 2) which encode two type-three secretion systems, T3SS-1 and T3SS-2

respectively [17, 18]. The T3SSs are multi-protein needle apparatuses that, once induced, are able to span the bacterial and eukaryotic membranes, translocating *Salmonella* effector proteins (effectors) into the host cell [18]. Using the T3SS-1 to invade epithelial cells, *S.* Typhimurium enters the host, replicates, and then induces inflammatory cell death of epithelial cells to release virulent *Salmonella* into the lumen [19]. It has also been shown in *S.* Typhimurium that the outer core of the lipopolysaccharide (LPS) is required for entry into intestinal epithelial cells [20].

Alongside epithelium invasion, *Salmonella* that was translocated across the intestinal epithelium interacts with immune cells in the lamina propria. The interaction of *Salmonella* LPS and flagella with polymorphonuclear cells (PMNs) induces a full inflammatory response with increases in expression of IL-6, IL-8, IL-1β, IL-17 and chemokines [6, 14]. *S.* Enteritidis is able to induce an increased expression of chemokines more than any other serovar in chickens and induce macrophage cell death with the T3SS-2 during the early stages of infection [17, 21]. In chickens, heterophils are the most important component of the gut innate immune system known to keep *S.* Enteritidis infection local and in low numbers. In most cases, *S.* Enteritidis manages to overcome PMN influx, infiltrate macrophages, and disseminate to the spleen, liver, and reproductive tract [6, 17, 22].

Once inside macrophages, *Salmonella* creates a replicative niche termed the *Salmonella* containing vacuole (SCV). The T3SS-2 has been implicated as the major regulator and maintenance mechanism for the SCV [23]. The SCV follows the host endocytic pathway while maturing with T3SS-2 effectors and other *Salmonella* stress

response mechanisms. T3SS-1 and T3SS-2 effectors induced while in the SCV, along with the formation of a *Salmonella* tubular network, prohibit the localization of the host lysosome and reactive oxygen species with the SCV [18, 23]. Without the lysosome and oxygen-dependent microbe-killing mechanisms, the macrophages cannot kill the *Salmonella* which prevents presentation to lymphocytes to activate the adaptive immune response. This has been seen in reduction of MHC expression and cytokine production in *S.* Typhimurium infection in dendritic cells, as well as in a murine infection model with *S.* Typhimurium that showed an inability to activate T-cells for an adaptive immune response [24, 25]. Without T-cell activation, low concentrations of IFNγ correspond to inactivated macrophages with decreased killing mechanisms leading to uninhibited replication of *Salmonella* in the SCV.

The SCV conditions include low concentrations of cations, acidic pH, and low nutrients. These conditions activate the T3SS-2 regulon as well as the RpoS, OmpR/EnvZ, and PhoP/PhoQ regulons [10, 26]. The PhoP/PhoQ regulon is specifically up-regulated in conditions of low Mg^{+2}/Ca^{+2} concentrations (such as inside macrophages), and includes multiple proteins involved in *Salmonella* outer membrane modulation, antimicrobial resistance, and virulence. Examples include: PgtE, an outer membrane protease able to cleave cationic antimicrobial peptides; YbjX, an unknown virulence protein; Mig-14, an antimicrobial resistance protein; and VirK, an antimicrobial resistance protein and virulence protein [10, 15]. Studies with PhoP/PhoQ regulated genes in the field have demonstrated that tools for specific innate immune defenses, such as antimicrobial activity, are also used by *Salmonella* for a wide range of

virulence functions. For example, in *Salmonella* serovar Typhi (*S.* Typhi), *mig-14* was found to be important for expression of invasion, virulence, flagellation, motility, and chemotaxis genes. In addition, after a mutation in *mig-14 S.* Typhi was less able to invade human epithelial HeLa cells compared to the wild type [27]. In another example, in *Shigella flexneri*, a *virK* mutant had an intercellular spreading defect. It was hypothesized in the study by Wing HJ, *et al.*, 2005 that the VirK protein alters the interaction between the outer membrane and IcsP (SopA), a protease, which negatively impacts IcsP (VirG). IcsP mediates assembly of a propulsive actin tail that allows for intercellular spread of the bacteria [16]. Also, in *S.* Typhimurium, *virK* and *somA* (*ybjX*) were found to be important in systemic spread as well as the later stages of infection [11].

Once *Salmonella* creates the SCV and establishes the replicative niche inside macrophages, transmission to other organs occurs as these macrophages travel through the lymphatic system. Among the serovars able to cause human salmonellosis, *S.* Enteritidis preferentially colonizes all parts of the reproductive tract of laying hens [28]. *S.* Enteritidis has a unique trait in that it can vary its outer membrane and its structures greatly, for a higher degree of heterogeneity, compared to other serovars [29, 30]. *S.* Enteritidis uses swarming based differentiation to induce hyper-flagellation, which increases the movement of *S.* Enteritidis within the host, possibly better than other serovars in mucous membranes. *S.* Enteritidis also has the capacity for quorum sensing, which allows it to activate various virulence proteins during different growth stages and organs in the host [29].

Establishment of *S.* Enteritidis in the reproductive tract leads to egg contamination. While *S.* Enteritidis is recovered at a higher frequency from the ovary than the oviduct, there is no difference in the percentage of recovery from yolk or albumen from infected eggs [28]. The yolk contains antibodies, whereas the albumen contains lysozymes, AvBDs, LPS binding proteins, ovotransferrin, and lacks iron. One theory to explain the higher deposition in egg albumen, even though ovarian *S.* Enteritidis colonization is higher, is that the genetic tools and stress responses available to *S.* Enteritidis make it more advantageous to combat these innate defenses to survive and replicate within the egg white [31]. One example is the increased isolation of high molecular weight LPS strains of *S.* Enteritidis from eggs. These strains have increased LPS glycosylation compared to *S.* Typhimurium and *S.* Enteritidis recovered from the environment. This high molecular weight LPS is also implicated in biofilm formation, or extracellular matrix formation, that is similar to the LPS capsule produced by *S.* Typhi to cause typhoid fever in humans [29].

Successful *S.* Enteritidis colonization involves inherent characteristics employed to subvert the reproductive innate immune system: phagocytes, antimicrobial peptides (including AvBDs, which are an important arm of chicken innate immunity), and immunoglobulins, without inducing overt inflammation and damage [31, 32]. Persistent reproductive tract colonization that leads to egg contamination is confined primarily to serovar Enteritidis, which is partially due to its ability to survive in these harsh conditions without causing overt clinical signs in the chicken host [29, 33]. While much information has been gathered on the significance and mechanisms of the T3SS-1 and 2,

the mechanisms of persistence employed by *S.* Enteritidis remain to be fully understood [18].

Most of our understanding of *Salmonella* pathogenicity is based on information gathered from *S.* Typhimurium experiments in mammalian hosts or cell cultures. Translation of data from *S.* Typhimurium to *S.* Enteritidis is not direct, especially since they have a 3% genetic difference, with the difference accounting for 6.4% of *S.* Enteritidis's genome and 9.6% of *S.* Typhimurium's genome. One major difference is in the composition of the outer membrane, a key barrier to innate defenses and interaction with the host [34, 35].The studies aimed at understanding the mechanisms of persistence in the chicken host often involve chicks. The data collected from these experiments do not transpose to an infection of mature hens with *S.* Enteritidis especially because the immunological landscape changes after point-of-lay [36]. In the present study we identified *S.* Enteritidis genes over-expressed in primary chicken oviduct epithelial cells and in chicken macrophages. From the genes identified, we characterized the antimicrobial peptide resistance (AMPR) genes *in vitro* and *in vivo* to elucidate the molecular mechanisms these genes use to evade the host defense mechanisms. The current investigation revealed that genes used by *S.* Enteritidis to evade host innate immune defenses also play a role in colonization and survival in the reproductive tract of laying hens and in egg deposition.

2. MATERIALS AND METHODS

2.1 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. Spontaneous nalidixic acid-resistant *S.* Enteritidis, designated ZM100, was generated by serial passages in Luria-Bertani broth (LB) with increasing concentrations of nalidixic acid. *S.* Enteritidis and *Escherichia coli* (*E. coli*) strains were cultured aerobically in tryptic soy broth (TSB), super optimal broth (SOB or SOC), LB broth, or on LB agar plates at 37°C. When appropriate, antibiotics were added at the following concentrations: chloramphenicol, 30 μg/ml; ampicillin, 100 μg/ml; nalidixic acid, 50 μg/ml.

Table 1. Bacterial strains and plasmids.

Strain or plasmid	**Description or relevant**	**Citation**
Strains		
S. enterica Enteritidis		
ZM100	Wild type *S.* Enteritidis, nal^R	[17]
ZM112	ZM100 nal^R, cm^R, *virK::*pEP185.2	This study
ZM114	ZM100 nal^R, cm^R, *ybjX::*pEP185.2	This study
ZM112C	ZM112 nal^R, cm^R, amp^R, pWSK*VirK*	This study
ZM114C	ZM114 nal^R, cm^R, amp^R, pWSK*YbjX*	This study
ZM122	ZM100 nal^R, *ΔvirK* (*Δ*14-919/930)	This study
ZM123	ZM100 nal^R, *ΔybjX* (*Δ*13-927/969)	This study
ZM124	ZM100 nal^R, *ΔvirKΔybjX* (Δ14-919/930)(Δ13-927/969)	This study

Table 1. Continued

Strain or plasmid	Description or relevant	Citation
Strains		
ZM122C	ZM122 nal^R, amp^R, pWSK*VirK*	This study
ZM123C	ZM123 nal^R, amp^R, pWSK*YbjX*	This study
E. coli		
S17-1	*recA, Tn7 λpir*	SZ collection
Top10F'	*F' lacQ*^{Q}*, Tn10(*ter^R*)*	Invitrogen
Plasmids		
pCR2.1	TA cloning vector, amp^R, kan^R, *lacZα*	Invitrogen
pEP185.2	Suicide vector, cm^R	[37]
pRDH10	Cm^R, *sacB*	[38]
pWSK29	Low copy expression vector, amp^R	[39]
pZM-16S	pCR2.1 16s rDNA	This study
pZM-23S	pCR2.1 23s rDNA	This study
pZM112	pEP185.2 carrying a fragment of *virK*	This study
pZM114	pEP185.2 carrying a fragment of *ybjX*	This study
pZM122	pRDH10 carrying the flanking regions of *virK*	This study
pZM123	pRDH10 carrying the flanking regions of *ybjX*	This study
pWSK*VirK*	pWSK29 carrying the *virK* gene	This study
pWSK*YbjX*	pWSK29 carrying the *ybjX* gene	This Study

2.2 Cell cultures and culture conditions

Primary chicken oviduct epithelial cells (COEC) were prepared as described previously [17]. Briefly, oviduct tissue (isthmus region) from 20-23 week old Hy-line W36 was obtained from a local poultry producer. After *Salmonella*-free status was confirmed by PCR, the tissue was washed extensively with Hanks balanced salt solution (HBSS) containing penicillin (200 U/ml) and streptomycin (200 mg/ml). After treatment with collagenase XI 1mg/ml (Sigma), the epithelial cells were retrieved by treatment with trypsin 0.25% in EDTA (Invitrogen), collected via centrifugation at 100 x g for 5 min, and resuspended in minimum essential media (MEM, Invitrogen) supplemented with 15% heat-inactivated fetal bovine serum (FBS), 2% chicken serum (CS), 0.05mM β-estradiol (Sigma), and 0.01mg/ml insulin (Sigma). COEC were seeded into 48-well tissue culture plates at a density of 4×10^4 cells per well (for SCOTS) or 96-well plates at a density of 2×10^5 cells per well (for invasion assays) and incubated at 37°C in 5% CO_2 for 48 h. The epithelial lineage was verified by immunofluorescent microscopy. Briefly, COEC were stained with monoclonal anti-pancytokeratin antibody and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG and examined with an Olympus IX81 FA scope. Cultures with more than 80% of cytokeratin-positive cells were used in subsequent infections.

HD11 chicken macrophage cells [40] were maintained in RPMI 1640 tissue culture medium (Invitrogen) supplemented with 10% FBS and 2% CS at 37°C in a 5% CO_2 atmosphere. Prior to infections, HD11 cells were seeded into 48-well tissue culture

plates at a density of 4 x 10^5 cells per well (for SCOTS) or 96-well plates at a density of 2 x 10^5 cells per well (for invasion assays) and incubated for 24 h.

2.3 Infection of cell cultures

Gentamicin protection assays were performed for invasion assays and selective capture of transcribed sequences (SCOTS) as described previously [41]. To prepare the bacterial inoculum, 50 µl of an overnight culture of *S.* Enteritidis strain (ZM100 only for SCOTS) was diluted into 5 ml of fresh TSB or LB broth and incubated aerobically at 37°C for 4 h, logarithmic phase, or 16 h, stationary phase. The *S.* Enteritidis cultures were harvested by centrifugation for 15 min at 1,500 × *g* and resuspended in fresh HBSS *S.* Enteritidis numbers from each inoculum were determined by measuring their optical density at 600 nm and confirmed by subsequent CFU enumerations by plating 10-fold serial dilutions.

Prior to infections, each cell culture, run in triplicate, was washed three times in their appropriate media containing no antibiotics. For SCOTS, 200 µL bacterial suspensions containing approximately 8 x 10^5 CFU of logarithmic phase ZM100 (for COEC) or 8 x 10^6 CFU stationary phase ZM100 (for HD11) were added into each well to reach a multiplicity of infection (MOI) of 20:1 (bacteria:cell). For invasion and intracellular replication assays, 200 µL bacterial suspensions containing approximately 2 x 10^7 CFU of either logarithmic or stationary phase *S.* Enteritidis strains (for both COEC and HD11) were added into the triplicate wells to reach a MOI of 20:1 for each well. To synchronize infections, all infected cell cultures were centrifuged at 800 x g for 10 min

and then incubated at 37^{o}C in a 5% CO_2 atmosphere for 1 h. Extracellular bacteria were removed by treatment with 100 µg/ml gentamicin in MEM (for COEC) or RPMI1640 (for HD11) at 37^{o}C in 5% CO_2 for 1 h. Following gentamicin treatment, infected cells were either lysed or maintained in fresh media containing 50 µg/ml gentamicin for an additional 3 and 15 h followed by lysis. These time points were designated 1 h post infection (hpi) (T1), 4 hpi (T4), and 16 hpi (T16), respectively. For RNA extraction, infected cells were lysed in Trizol (200 µl/well). For invasiveness and intracellular replication studies, infected cells were lysed in 0.5% Triton X-100 (100 µL/well). Then, ten-fold serial dilutions of the invaded cell lysates were plated onto LB agar supplemented with appropriate antibiotics and incubated overnight at 37^{o}C for CFU enumeration. Invasiveness for each strain was calculated as the proportion of inoculum internalized at T1, and the intracellular replication, or survival, was calculated as the proportion of *S.* Enteritidis recovered at T4 or T16 to the inoculum.

2.4 Preparation of bacterial genomic DNA and rDNA

Genomic DNA (gDNA) was isolated from overnight ZM100 culture using the Blood & Cell Culture DNA Midi Kit according to the manufacturer's instruction (Qiagen). Biotinylation of gDNA was carried out by mixing equal amounts (20 µg) of gDNA and photosensitive biotin (Sigma) in a final volume of 50 µl inside a 0.5 ml tube; the tubes were exposed to strong incandescent light (200 W) for 30 min. The labeled gDNA was extracted with 2-butynal, washed with 70% ethanol, and resuspended in 10mM Tris-HCl/0.5mM EDTA (1x TE) buffer.

The 16S and 23S rRNA coding regions of *S.* Enteritidis were amplified by PCR using primers, 16s-F1/R1 and 23s-F1/R1, respectively (Table 2). The PCR products were cloned into pCR2.1 (Invitrogen). The resulting plasmids, pZM-16S and pZM-23S, were propagated in *E. coli* TOP10F' (Invitrogen). Plasmid DNA was extracted using the Wizard® Plus Minipreps DNA purification system (Promega) according to the manufacturer's instruction. DNA concentration was determined based on the A260 spectrophotometer reading.

2.5 Isolation of RNA

Total RNA was isolated from ZM100-infected cell cultures and ZM100 grown in TSB using the Trizol reagents per the manufacturer's instruction (Life Technologies). RNA samples were treated with RNase-free DNase I (Ambion) according to the manufacturer's instructions. RNA concentration and integrity were determined by A260/A280 spectrophotometric readings and agarose gel electrophoresis.

2.6 Synthesis of cDNA

RNA (5 µg) was converted to first strand cDNA by random priming with the Superscript III enzyme (Invitrogen) according to manufacturer's instruction. Random-priming was achieved by using primers ZM1-Nw and ZM2-Nw with 9 random nucleotides at their 3'-ends for intracellular bacteria and broth-grown bacteria, respectively. Double-stranded cDNA was generated using the Klenow DNA polymerase (Promega) according to manufacturer's instruction. The double-stranded cDNA was then

amplified by PCR using primers ZM1-Nw (for intracellular bacteria) or ZM2-Nw (for broth-grown bacteria) without the 3' random nucleotides. PCR was performed using Platinum®Taq DNA Polymerase (Sigma-Aldrich) under the following conditions: initial denaturation of 3 min at 94°C followed by 25 cycles at 94°C for 45 s, at 58°C for 45 s, at 72°C for 2 min, and a final elongation at 72°C for 10 min. The amplified cDNA was precipitated in 100% ethanol (2.5 v/v) with 3 M NaOac (0.1 v/v) and 1 µl Glycogen (1 µg/ml), and resuspended in 10 mM N-(2-hydroxyethyl) piperzine-N'-3-propanesulfonic acid/1 mM EDTA (1x EPPS/EDTA). DNA concentration was determined based on the A260 spectrophotometric reading.

2.7 Selective capture of transcribed sequences (SCOTS)

To block rRNA coding regions, pZM-16S, pZM-23S (cloned rDNA), and biotinylated gDNA were mixed at a ratio of 5:5:1 (µg), fragmented by sonication, precipitated, and resuspended in (1x EPPS/EDTA). The DNA mixture was divided into aliquots (0.3 µg gDNA, 3 µg rDNA in 8 µl) and stored at -80°C prior to hybridization. The rDNA-blocked gDNA (8 µl) and the amplified cDNA (3 µg in 8 µl 1x EPPS/EDTA) were denatured separately at 98°C for 3 min. Following addition of 2 µl 5M NaCl to each reaction tube, the denatured gDNA and cDNA were self-annealed at 67°C for 30 min. Then the gDNA and cDNA were mixed and hybridized at 67°C for 20 h. The cDNA molecules hybridized to the biotinylated gDNA were captured by incubation with streptavidin-coated beads and subsequent elution according to the manufacturer's instruction (Dynal). The eluted cDNA was amplified by PCR using specific primer ZM-

1 for intracellular bacteria or ZM-2 for extracellular bacteria. For each time point/cell type/growth condition combination, 10 parallel hybridization reactions were performed. The amplified cDNA from the 10 reactions were combined and subjected to another round of hybridization. Three rounds of hybridizations were carried out to enrich the cDNA species representing S. Enteritidis gene transcripts at a given time in a given growth condition. Following enrichments, competitive hybridizations were performed: rDNA-blocked and biotinylated gDNA were prehybridized with cDNA derived from broth-grown bacteria at 67°C for 4 h and then hybridized with cDNA of intracellular bacteria for additional 20 h. The hybridized cDNA specific to intracellular bacteria was captured using streptavidin-coated beads and amplified using primer ZM-1. Three rounds of competitive hybridizations with 10 parallel reactions in each round were performed to enrich the transcripts specific to intracellular bacteria. The cDNA specific to intracellular bacteria at each time point post infection of COEC or HD11 was cloned into pCR2.1. Following transformation of *E. coli* TOP10F', all clones with inserts were selected and sequenced commercially (Operon-MWG). The sequence of each insert was compared to the *S. enterica* genomes using the BLASTN algorithm. The SCOTS procedures were performed twice for each type of cells using RNA derived from two independent infections.

2.8 Reverse transcriptase polymerase chain reaction

Reverse transcriptase polymerase chain reaction (RT-PCR) was conducted using MultiScribe reverse transcriptase (Invitrogen) and the SYBR® Green PCR Master Mix

(Applied Biosystems) according to the manufacturer's instructions. The primer sequences of *Salmonella* genes were obtained from the Entrez Nucleotide database and listed in Table 2. Reverse transcription of total RNA (2 μg) in a volume of 100 μl containing 5.5 mM MgCl2, 500 μM dNTP, 2.5 μM random hexamers, and 1.25 U of MultiScribe reverse transcriptase was performed at 42^{o}C for 30 min. The resultant cDNA product was used as a template (4 μl / reaction) for subsequent real-time PCR (ABI Prism 7700, Applied Biosystems). PCR was carried out in a volume of 25 μl under the following conditions: 95°C for 10 min followed by 45 amplification cycles of 95°C for 15 s, and 58°C for 30 sec, and 72°C for 30 sec in the presence of 1 x SYBR® Green PCR Master Mix (Applied Biosystems). To quantify the elevated transcription of genes within host cells, the amount of 16s rDNA was used to normalize the cDNA concentrations of different samples. The normalized amount of transcripts in intracellular bacteria relative to the amount of transcripts in broth-grown bacteria at each time point was calculated as fold-change using the formula $2^{-\Delta\Delta Ct \pm SD}$ where SD is the standard deviation [42].

Table 2. Primers used in this study[A].

Primer	Sequence (5' – 3')	Amplicon size (bp)
Primers for SCOTS		
ZM-1	GACACTCTCGAG ACATCACCGG	N/A
ZM-2	TGCTCTAGACGTCGACATGGTT	
ZM1-Nw	GACACTCTCGAGACATCACTGG(N_9)	N/A
ZM2-Nw	TGCTCTAGACGTCGACATGGTT(N_9)	
16s-F1	CGGACGGGTGAGTAATGTCT	1,372
16s-R1	ATCACAAAGTGGTAAGCGCC	
23s-F1	CGGGGGAACTGAAACATCTA	2,636
23s-R1	TCAACGTCGTCGTCTTCAAC	

Table 2. Continued

Primer	Sequence (5' – 3')	Amplicon size (bp)
Primers for real-time PCR		
16srRNA-F	CCTTACGACCAGGGCTACACACG	94
16srRNA-R	GGACTACGACGCACTTTATGAGG	
hsdS-F	TTGAAAAGACAATCCCACTC	120
hsdS-R	GGTAACCAACAACTCCCG	
orgAa-F	AACGGATAAACTTGTTCCCTGAT	110
orgAa-R	TCGGTTGCCATAAACTGAG	
pgtE-F	AACTGGACTGGAAAATAAAAAATGT	120
pgtE-R	TATGACCCGATCCCGACG	
pipB-F	TCGGTGCAAATTTGTGTTGT	142
pipB-R	GAGCCGAATAGAATTGCAGC	
prgJ-F	GAAAAAGCCTGGAGTAGCC	95
prgJ-R	GTCCCTGAGAATGCCGTT	
prgK-F	ACGCCCTCCATCGTCTGT	93
prgK-R	TTCGCTGGTATCGTCTCC	
sefB-F	CTCCATTTATTGTAACACCACCTAT	123
sefB-R	TTACACACAACCAATACAAAGACTC	
ssaD-F	ATCCAAATAAGCCGCTACCA	84
ssaD-R	CAAGTTCACAATCCTGTTTACCAA	
ssaK-F	CTGTTCCAGCCATTCCACTTCCAT	113
ssaK-R	TCATCCGAGACGCCTATCGTTATCA	
ssaI-F	TGCCTGTAAGCACTCAATCT	125
ssaI-R	CTGCGGTAATAAAGCACTGG	
ssaJ-F	CGTCTCAGGCAAAAATAGC	118
ssaJ-R	ACGCCAATAAAGGGAAGG	
sthC-F	ATTCAGCCCTGACCACCG	128
sthC-R	ACCTTATGCTTCGCCTTACCA	
yifK-F	CGTGGGCGAACTATTTGA	131
yifK-R	AACTTTGTAGACCAGCGTGA	
yjjZ-F	GCGTATTATTGCCTGGAGTGAT	132
yjjZ-R	AAAATGCCGTAATTGTTTGTGAT	
ybjX-F	GACGATGTAGCCCGAATAGG	81
ybjX-R	TACTGACCAATCTCACCCAAT	
virK-ORF1	GCGAGCTCATGACGATGCAGCAAAG	138
virK-R	AATAAGGCAACGTAATAC	

Table 2. Continued

Primer	Sequence (5' – 3')	Amplicon size (bp)
Primers for mutant construction and complementation		
virK-ORF1	GCGAGCTCATGACGATGCAGCAAAG	
virK-R	AATAAGGCAACGTAATAC	
ybjX-ORF1	GCGAGCTCATGTCGCGGATTACGAT	
ybjX-rt-R	CGACGAAAGCTGGCTTTAC	
virK-UF1	CTGGCTTACACAATAGCAG	
virK-UR1	ACTCTAGAGCTGCATCGTCATACTAC	
virK-DF1	ACTCTAGATCTCCCGGTAGAACTATTTC	
virK-DR1	CTGGGTGCATATTGATAC	
ybjX-DF2	TATGGGAATCGAGTGG	
ybjX-DR2	ACTCTAGAGATAGCGTCGTCGAAC	
ybjX-UF2	ACTCTAGAAATCCGCGACATAAGA	
ybjX-UR2	ATCTGGGTCAATCACG	
ybjX-ORF1	GCGAGCTCATGTCGCGGATTACGAT	
ybjX-ORF2	GCTCTAGATTAACGTTTGAATGTGAC	
virK-ORF1	GCGAGCTCATGACGATGCAGCAAAG	
virK-ORF2	GCTCTAGACTACCGGGAGAGGCTGTTA	

[A]The restriction sites integrated into the sequences are underlined.

2.9 Construction of mutants

Mutants and complemented strains were constructed using the primers listed in Table 2. Initial insertion mutants, *virK* (ZM112) and *ybjX* (ZM114), were constructed for inactivation of the target gene as previously described [43]. Briefly, DNA fragments encoding the 5'-termini of *virK* and *ybjX* were amplified by PCR using primer pairs, virK-ORF1/R, and ybjX-ORF1/rt-R, respectively. The PCR products were cloned into pCR2.1TOPO (Invitrogen, USA). The inserts were then excised from pCR2.1TOPO with SacI and XbaI and subcloned into the corresponding sites of pEP185.2, a suicide vector coding for chloramphenicol resistance [37]. The resulting plasmids, pZM112 and

pZM114, were introduced into *E. coli* strain S17-1λ*pir* by chemical transformation and transferred into strain ZM100 by conjugation. Ex-conjugants with a pEP185.2 insertion into the chromosome of strain ZM100 were selected by growth on LB agar plates supplemented with chloramphenicol and nalidixic acid. Inactivation of each gene was confirmed by DNA sequencing.

To complement the genetic defect associated with inactivation of *virK* and *ybjX*, the open reading frames of these genes were amplified by PCR using primer pairs VirK-ORF1/ORF2, and YbjX-ORF1/ORF2. The PCR products were cloned directionally under the *lac* promoter of pWSK29, a low copy number expression vector [39], which generated plasmids pWSKVirK and pWSKYbjX. The correct orientation of each target gene in pWSK29 was confirmed by DNA sequencing. These plasmids were introduced into the corresponding mutant strains by electroporation and selection for resistance to ampicillin. The resultant strains were designated as ZM112C and ZM114C, respectively.

To avoid possible polar effects, unmarked deletion mutants Δ*virK* (ZM122), Δ*ybjX* (ZM123) and a double mutant Δ*virKΔybjX* (ZM124)) were constructed using allelic exchange mutagenesis as previously described [43]. Briefly, the upstream and downstream regions of the genes were amplified by PCR using primer pairs, virK-UF1/UR1, virK-DF1/DR1, ybjX-UF2/UR2, and ybjX-DF2/DR2, respectively. After ligation of the upstream and downstream products, the fusion was re-amplified by PCR and cloned into pCR2.1TOPO. The fusion was then sub-cloned into vector pRDH10, a suicide vector carrying the *sacB* and chloramphenicol resistance genes as well as being λ*pir*-dependent [38]. The resulting plasmids, pZM122 and pZM123, were chemically

transformed into *E. coli* S17-1λ*pir*, selected by resistance to chloramphenicol, then transferred to ZM100 by conjugation. Ex-conjugants were selected by resistance to nalidixic acid and chloramphenicol for the insertion of the plasmid in to the genome. To select for second recombination event that would remove the plasmid and result in the unmarked deletion, selection was done in 10% sucrose LB broth, followed by growth on 5% sucrose LB plates at 30^{o}C. The colonies that were sensitive to chloramphenicol were subjected to PCR to screen for the deletion of interest. Each unmarked deletion was complemented as above with their respective pWSK29 plasmids harboring the ORF of the gene. The unmarked deletion for each gene was confirmed by DNA sequencing.

2.10 Bacterial cell morphology assay

Bacterial morphology for ZM100, Δ*virK*, Δ*ybjX*, Δ*virKΔybjX*, Δ*virK* pWSK*VirK* (ZM122C) and Δ*ybjX* pWSK*YbjX* (ZM123C) was determined by microscopic examination of logarithmic and stationary cultures at 4 h and 16 h growth in LB broth at 37^{o}C and 250 rpm. Three fields were captured at 40x from three separate experiments by a Sony microscope camera and analyzed for average Feret length using ImageJ morphometric analysis [44]. After conversion to micrometers (12.156 pixels/μm), the data are presented as the average length per strain per time point.

2.11 Cell motility assay

To test the ability of ZM100, Δ*virK*, Δ*ybjX*, Δ*virKΔybjX*, Δ*virK* pWSK*VirK* and Δ*ybjX* pWSK*YbjX* strains to swim, bacteria were inoculated into 0.3% agar LB plates as

previously described [45]. Briefly, equal amounts of logarithmic (4 h) cultures were spotted in the middle of 0.3% agar plates and incubated at 37^{o}C for 3 h at which their motility diameter was measured. The data are represented as the relative motility (% of ZM100).

2.12 EDTA and deoxycholic acid sensitivity assays

ZM100, Δ*virK*, Δ*ybjX*, Δ*virKΔybjX*, Δ*virK* pWSK*VirK* and Δ*ybjX* pWSK*YbjX* were tested for their sensitivity to EDTA and deoxycholic acid (DOC) as previously described [46, 47]. Briefly, approximately 1 x 10^{8} CFU bacteria were added to warm 0.5% LB agar which was poured over 1.5% LB agar plates. Once dried, filter disks containing 0.5M EDTA were placed in the center of the agar and incubated without inversion at 37^{o}C for 16 h. The zones of inhibition were measured in millimeters (mm) and the data are presented as the relative sensitivity (% difference to ZM100). For the DOC sensitivity, LB agar plates containing 1% DOC and plain LB agar plates were inoculated with ten-fold serial dilutions of bacterial culture and incubated overnight at 37^{o}C for enumeration. Percent inhibition of growth was calculated using the formula: [(CFU on plain LB agar – CFU on 1% DOC LB agar)/ (CFU on plain LB agar)] x 100.

2.13 Cell supernatant 2D SDS-PAGE assay

Cell supernatant proteins were obtained from ZM100, Δ*virK*, Δ*ybjX*, and Δ*virKΔybjX* as previously described [48]. Briefly, bacterial cultures were grown in triplicate overnight at 37^{o}C and 250 rpm, subcultured 1:50x into fresh LB broth and

incubated 4 h at 37^{o}C and 250 rpm. Culture supernatants from approximately 12 x 10^{9} CFU for each strain were recovered by centrifugation at 5,000 x g at 4^{o}C for 15 min and filtration through a 0.45-µm-pore-size sterile filter. The supernatant was concentrated using 3 K molecular weight cutoff centrifugal filters, and the proteins in the supernatant were obtained by methanol-chloroform protein precipitation. A portion of the total proteins recovered was visualized on a 12.5% sodium-dodecyl sulfate polyacrylamide gel (SDS-PAGE) stained with 0.1% silver stain to check for purity. The proteins were then analyzed by 2D SDS-PAGE. Seven-centimeter non-linear isoelectric focusing strips, pH 3-10, were rehydrated overnight with proteins dissolved in UT Chaps buffer (7 M Urea, 2 M Thiourea, 4% chaps) with DeStreak, pharmalytes, amylytes, and bromo-phenol-blue (GE Healthcare), subjected to 770 volt-hours, then separated on a 12.5% SDS-PAGE gel and stained with 0.2% silver stain. After three independent experiments were completed, densitometric analysis with ImageJ determined the difference in spot densities between the deletion mutants, Δ*virK*, Δ*ybjX*, and Δ*virK*Δ*ybjX*, and wild type ZM100 [44]. Spots that were greater or less than 30% of the density of ZM100 were excised, reduced and alkylated with iodoacetamide, digested with trypsin, and then ran on a ThermoFisher LTQ or OrbiTrap linear ion trap mass spectrometer using nano-LC peptide separations. Proteins were analyzed with Scaffold 4.1 for specific protein identities by blasting to NCBI and UniProt databases [49].

2.14 Avian beta-defensin sensitivity assay

The mature peptide of Avian beta-defensin-6 **(**AvBD6) (SPIHACRYQRGVCIPGPCRWPYYRVGSCGSGLKSCCVRNRWA) was custom synthesized, purified and confirmed at LifeTein LLC (Hillsborough, NJ), the disulfide bridge pairing with Cys1-Cys5, Cys2-Cys4, and Cys3-Cys6. The purity (>98%) of the synthetic peptide was determined by mass spectrometry >98% following reverse-phase high-performance liquid chromatography (HPLC). Synthetic AvBD6 was diluted in sterile distilled water prior to use. The sensitivity of *S.* Enteritidis strains, ZM100, *virK*, *ybjX*, *virK* pWSK*VirK* (ZM112C), and *ybjX* pWSK*YbjX* (ZM114C) to avian β-defensin (AvBD)-6 was determined using a micro-broth dilution method as described previously [50]. In brief, overnight cultures were diluted in fresh Muller Hinton broth to achieve a 0.5 McFarland turbidity standard (approximately 5 x10^7 CFU/ml). Equal volumes (50 μl) of bacterial suspension and synthetic AvBD-6 (32 μg/ml) were mixed and incubated at 37ºC for 1 h. For controls, PBS was used to replace AvBD-6. Following incubation, serial dilutions of each culture were prepared in PBS and plated on LB agar plates for CFU enumeration. AvBD sensitivity was expressed as percent of growth inhibition relative to the growth of the AvBD-free control: [100 x (CFU of PBS-treated culture – CFU of AvBD-treated culture)/CFU of PBS-treated culture].

2.15 Polymyxin B sensitivity assay

S. Enteritidis strains, ZM100, *virK*, *ybjX*, *virK* pWSK*VirK*, and *ybjX* pWSK*YbjX* , were grown at 37°C in N-minimal media containing 10 mM MgCl as described

previously [51]. The pH of the medium was buffered with 100 mM Tris-HCl, pH 7.4. Stationary cultures were diluted 1:100 in pH 7.4 10 mM MgCl and incubated for 3 h at 37°C. Approximately 5 x 10^4 bacteria from each culture were inoculated into LB containing 2.5 µg/ml polymyxin B (Sigma) and incubated at 37°C for 1 h. Serial dilutions of each culture were prepared in PBS and plated on LB agar plates for CFU enumeration. Polymyxin B sensitivity was expressed as log Reduction: [log Input (CFU/ml) – log Viability (CFU/ml)].

2.16 Animal experiments

Animal rearing, maintaining, and euthanasia were performed according the recommendations by the Guide for the Care and Use of Laboratory Animals of the National Institute of Health and to our Texas A&M University Institutional Animal Care and Use Committee SACC approved Animal Use Protocol (Permit number: 2011-143).

Female Hy-Line W36 chicks, obtained at 1 d old from a local hatchery without vaccination for *S.* Enteritidis, were maintained at 30^{o}C till they were 7 weeks old, and then moved to floor pens at room temperature till they were 21 weeks old. They were given feed (standard chick diet and standard layer diet, respectively) and water *at libitum*. Prior to experiments, three hens were sacrificed and their spleen, oviduct, ovary, and cecum were tested for the presence of *Salmonella* by PCR.

For the infection challenge, the hens were housed in an ABSL-2 housing facility with two hens per cage and grouped into six cage stacks so that seven sets of twelve hens were isolated from each other. While on a 16 hour light and 8 hour dark schedule the

hens were given water and standard layer diet *ad libitum* for the duration of the infection experiment. The bacterial inoculum was prepared by having the desired bacterial strains (ZM100, Δ*virK*, Δ*ybjX*, Δ*virK*Δ*ybjX*, Δ*virK* pWSK29*VirK* and Δ*ybjX* pWSK29*ybjX*) cultured in LB broth overnight at 37^{o}C and 250rpm. The subsequent cultures were diluted 1:50x into sterile LB broth and incubated for 16 h at 37^{o}C and 250 rpm. Resultant cultures were harvested by centrifugation at 3,000 rpm at 4^{o}C for 20 min and resuspended in sterile saline at a final concentration of 5 x 10^{9} CFU/ml. Then, each group of twelve hens was orally inoculated with one ml per hen of one bacterial strain; one group of hens was inoculated with one ml of sterile saline to act as a control. At the same time each day post infection (dpi) all viable eggs and a fecal sample were collected from each cage. From 1 dpi to 5 dpi, the fecal and egg samples represented two hens; from 6 dpi to 10 dpi, the fecal and egg samples represented one hen. At 5 dpi and 10 dpi, six hens from each group were humanely euthanized and the spleen, oviduct (isthmus region), ovary, cecum, and intestine were collected and stored at -80^{o}C.

For bacterial enumeration, approximately one gram of feces was resuspended in10 ml of buffered peptone water (BPW) and ten-fold serial dilutions were plated onto XLT4 agar followed by incubation at 37^{o}C for 16 h. The BPW was enriched overnight at 37^{o}C and plated on XLT4 in which any growth was arbitrarily assigned 5 CFU/g, the detection threshold for that procedure. The collected eggs were washed three times in 70% ethanol to remove exterior bacteria. Cleaned, whole eggs were placed in 50 ml of BPW and stomached for 5 min. A portion of the stomached egg-BPW mixture was plated onto selective LB agar for enumeration and the remainder was enriched for two

days at 37^{o}C with plating 24 h and 48 h enrichments on selective LB agar. Tissue was thawed and samples from each tissue were aseptically collected, weighed, homogenized for 30 sec in 5 ml PBS, and plated on selective LB for strain enumeration. The cecum and intestine contents were separated from the tissue, which was washed three times in PBS before homogenization and plating. Homogenate broths were enriched overnight at 37^{o}C and plated on selective LB agar. Cultures that were negative in initial plating but that had growth after enrichment were arbitrarily assigned 2.5 CFU/ homogenate for the spleen, ovary, and oviduct tissue and 12.5 CFU/ homogenate for the cecum contents. The detection thresholds for the different types of samples were arithmetically determined using the following formulas: (arbitrary tissue CFU/homogenate = [((1 CFU / 0.5 ml) x 5 ml) / 4], arbitrary cecum content CFU/homogenate = [((1 CFU/ 0.1 ml) x 5 ml) / 4]). Three positive colonies, when possible, were tested by PCR from each positive culture to validate accuracy of the visual counts. The data are expressed as log CFU/g for the feces and tissue, except the ovary which is expressed as log CFU/ovary.

2.17 Statistical analysis

After normality was confirmed, one-way ANOVA statistical analysis was run to determine significant differences among the groups in the different experiments and Student's *t*-test was used to determine any significant differences between the individual strains tested in each experiment ($p<0.05$).

3. RESULTS

3.1 Detection of *S.* Enteritidis genes over-expressed in infected chicken cells by SCOTS

To better understand the mechanism of *S.* Enteritidis colonizing chickens, SCOTS procedures were performed to identify the genes preferentially expressed in a chicken macrophage cell line (HD11) and primary chicken oviduct epithelial cells (COEC), two main cell types utilized by *S.* Enteritidis for systemic infection and reproductive tract colonization. Following three rounds of enrichment for cDNA molecules derived from intracellular and broth-grown bacteria and three rounds of competitive hybridizations, the cDNA representing *S.* Enteritidis genes transcribed in COEC or HD11 were cloned and sequenced. For each type of cells at each time point, only the transcripts identified by two independent SCOTS procedures following two infections were considered as intracellularly expressed. Using this stringent selection criteria, a total of 48 genes were identified (Table 3). Of those genes, 37 were over-expressed in COEC, 26 in HD11, and 15 in both types of cells.

For a selected group of genes, intracellular expression was further confirmed by quantitative real-time PCR using 16s rRNA as a reference gene as shown in Fig 1. The genes specific to COEC consisted of those encoding SPI-1 T3SS components, restriction modification enzymes, oxidative stress resistance, proteins involved in fimbrial biogenesis and outer membrane assembly. The elevated transcription of COEC-specific genes occurred mainly at 1 hpi. The genes expressed within HD11 cells at 1 hpi or 4 hpi

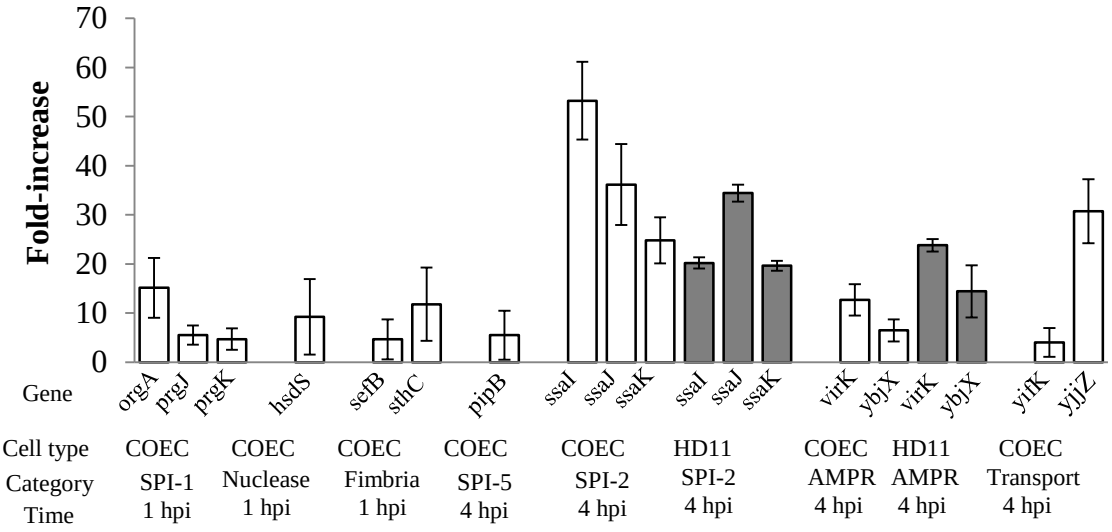

Figure 1. Quantitative analysis of genes over-expressed by *S.* Enteritidis upon infection in COEC and HD11 cells. Relevant genes found to be over-expressed in COEC or HD11 cells from the SCOTS experiment were quantified for their intracellular expression in these cell types using reverse transcriptase real-time PCR; the fold increase is shown ($2^{-\Delta\Delta Ct \pm SD}$ where SD is the standard deviation). For each gene shown, the cell type, category, and time post infection in which the expression occurred are shown below the graph. Assays were performed in duplicate three separate times.

Table 3. Genes over-expressed by *S.* Enteritidis in primary chicken oviduct epithelial (COEC) and macrophages (HD11).

			COEC		**HD11**	
Category	**Gene**	**Description and possible function**	**1h**	**4h**	**1h**	**4h**
Virulence, SPI-1	*orgA*	Type III secretion, host cell invasion	+			
	prgJ	Type III secretion, host cell invasion	+			
	prgK	Type III secretion, host cell invasion	+			
Virulence, SPI-2	*ssaD*	Type III secretion, intracellular survival		+		+
	ssaI	Type III secretion, intracellular survival		+		+
	ssaJ	Type III secretion, intracellular survival		+		+
	ssaM	Type III secretion, intracellular survival		+		+
	ssaK	Type III secretion, intracellular survival		+		+
Virulence, SPI-5	*pipB*	T3SS-2 secreted protein		+		+

Table 3. Continued

Category	Gene	Description and possible function	COEC		HD11	
			1h	4h	1h	4h
Antimicrobial peptide resistance	*pgtE*	Outer membrane protease E	+		+	
	virK	Intracellular survival	+		+	
	ybjX	Putative virK homologue	+		+	
Membrane transport, metabolism, stress response	*fumA*	Fumarate hydrolase	+			
	rpiA	Ribose 5-phosphate isomerase	+			
	yliG	Putative Fe-S oxidoreductase	+			
	ahpC	alkyl hydroperoxide reductase, oxidative stress resistance		+		
	acs	Acetyl-coenzyme A synthetase		+		
	gip	Glyoxylate-induced protein,		+		
	hemX	Uroporphyrinogen III methylase		+		
	hyi	Hydroxypyruvate isomerase		+		
	gilnD	Uridylyltransferase			+	
	narU-Y	Respiratory nitrate reductase, nitrite extrusion protein			+	
	pheA	Prephenate dehydratase				+
	ybbP	Putative inner membrane ABC transporter			+	
	yiaH	Putative inner membrane protein,			+	
	sb35	Hydrolase of HD superfamily		+		
	yifK	Putative ABC transporter		+	+	
	yfdZ	Putative aminotransferase	+		+	
	yin-cysE	Putative mandelate racemase/muconate lactonizing enzyme, serine acetyltransferase	+			+
	yraO-P	Putative phosphoheptose isomerase		+		
	trpS	Tryptophanyl-tRNA synthetase		+		+

Table 3. Continued

Category	Gene	Description and possible function	COEC		HD11	
			1h	4h	1h	4h
Membrane, transport, metabolism, stress response	*tdcB*	L-threonine/ L-serine permease, anaerobically inducible				+
	tdcC-D	catabolic threonine dehydratase, anaerobic metabolism				+
Restriction modification	spa1514	Putative DNA/RNA non-specific endonuclease	+			
	hsdM	DNA methylase, protect DNA against endonuclease	+			
	hsdS	DNA methylase, protect DNA against endonuclease	+			
	hsdR	DNA methylase, protect DNA against endonuclease	+			
Cell wall and surface structure	*sefB*	Fimbrial periplasmic chaperon, pili assembly, adherence	+			
	sthC	Fimbrial usher protein, adherence	+			
	murC	UDP-N-acetylmuramate:alanine ligase, cell wall synthesis			+	
	mpl	Murein peptide ligase, cell wall synthesis				+
	gtrB	Glucosyl transferase, O-antigen conversion				+
	yjjZ	Inner membrane protein, function unknown		+	+	
	yfiO	Lipoprotein, outer membrane assembly	+			
Transcription	*arcB*	Aerobic respiration control sensor, global regulation	+			
	nusB	Transcription antitermination	+		+	+
	rpoN	RNA polymerase sigma-54, nitrogen assimilation		+		

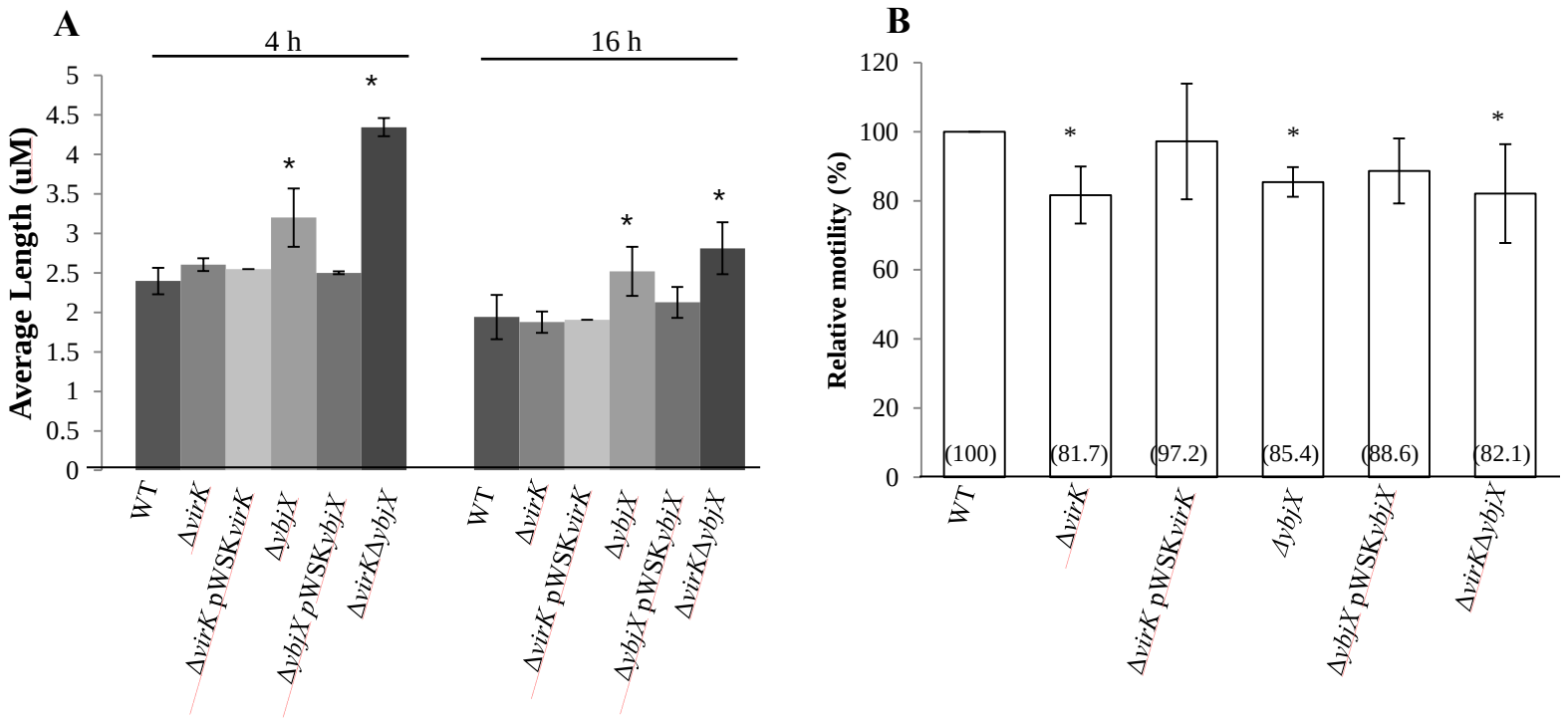

Figure 2. Average length and motility of AMPR mutants. Wild type *S.* Enteritidis (WT, ZM100), Δ*virK* (ZM122), Δ*ybjX* (ZM123), Δ*virKΔybjX* (ZM124), Δ*virK* pWSK*VirK* (ZM122C), and Δ*ybjX* pWSK*YbjX* (ZM123C) were used to determine average length and relative motility. **A.** *S.* Enteritidis strains were grown to log phase (4 h) or stationary phase (16 h), photographed at 40x magnification, and analyzed for average feret length using ImageJ morphometric analysis. Strains Δ*ybjX* and Δ*virKΔybjX* exhibited increased average lengths in both phases of growth. **B.** Log phase *S.* Enteritidis strains were inoculated onto 0.3% agar, incubated at 37^0C , and then had their motility diameter measured (mm). All three AMPR mutants had decreased swimming diameters. The results are shown as the percent of the distance WT traveled after 3 h incubation with the percent for each strain noted in parentheses. All experiments were run in duplicate at least three separate times.* denotes statistical significance ($p<0.05$).

included those involved in nitrate reduction, cell wall synthesis, and O-antigen conversion, as well as the anaerobically induced *tdc* operon. *S.* Enteritidis genes over-expressed in both cell types comprised those encoding SPI-2 T3SS apparatus, SPI-5 encoded *pipB,* and genes responsible for antimicrobial peptide resistance (AMPR): v*irK* and *ybjX*. The increased expression of the genes in both cell types was detected at 4 hpi, suggesting the significance of these genes in intracellular survival or replication inside COEC and HD11. In addition, several genes of unknown function were over-expressed in both types of cells, such as *yifK* and *yjjZ,* hypothetically involved in transport, with the latter being over-expressed around 30-fold (Fig. 1).

3.2 S. Enteritidis ΔybjX and ΔvirKΔybjX mutants have altered cell morphology

To characterize the functional contributions of AMPR genes to S. Enteritidis pathogenicity, we constructed unmarked deletion mutants of *virK* and *ybjX*. The differences in morphology between the wild type *S.* Enteritidis (ZM100) and Δ*virK* (ZM122), Δ*ybjX* (ZM123), and Δ*virKΔybjX* (ZM124) mutant strains were examined microscopically. During logarithmic phase and stationary phase of growth (4 h and 16 h respectively) Δ*ybjX* and *ΔvirKΔybjX* mutants formed long filaments as evidenced by their longer average length (µm) and chain forming morphology when compared to *S.* Enteritidis ZM100 (WT) (Fig. 2A, Fig. 3C, and Fig. 3D; $p<0.05$). At logarithmic phase, the *ΔvirKΔybjX* mutant exhibited the longest length, 4.3 µm, and most chain forming cells when compared to all strains. Wild type morphology was restored to the Δ*ybjX* mutant when cloned *ybjX* gene (pWSKYbjX) was introduced into the strain (Fig. 2A,

Fig. 3E). In contrast the Δ*virK* mutant had no difference in length or morphology compared to the wild type. From these observations, we conclude that *ybjX* contributes to maintaining normal, rod shape morphology, and that *virK* may contribute to cell morphology through a different mechanism, accountable for the increased difference in morphology in the Δ*virKΔybjX* mutant.

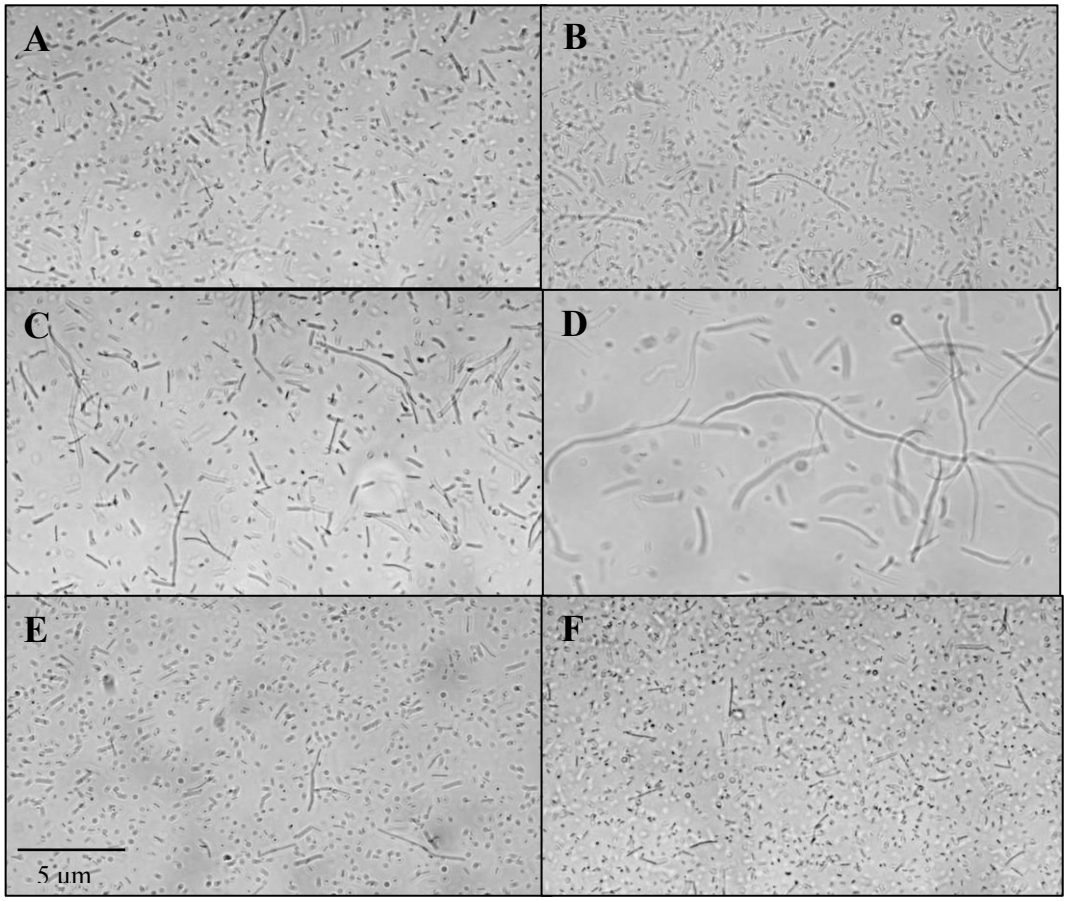

Figure 3. Cell morphology of AMPR mutants. The cell morphology of log phase growing *S.* Enteritidis wild type , AMPR mutants, and their complements was observed under 400x magnification. Wild type, Δ*virK* , and the complemented strains (Δ*virK* pWSK*VirK* and Δ*ybjX* pWSK*YbjX*) displayed typical short rod morphology. Mutant Δ*ybjX* and Δ*virKΔybjX* had elongated cells with the double mutant exhibiting long chain formations. Representative pictures are shown: **(A)** Wild type S.E. (ZM100), **(B)** Δ*virK* (ZM122), **(C)** Δ*ybjX* (ZM123), **(D)** Δ*virKΔybjX* (ZM124), **(E)** Δ*virK* pWSK*VirK* (ZM122C), and **(F)** Δ*ybjX* pWSK*YbjX* (ZM123C).

3.3 *S.* Enteritidis AMPR mutants have decreased motility

Additional experiments were carried out to determine if mutations in *virK* and *ybjX* alter motility in *S.* Enteritidis. To assess this, we examined the ability of the wild type *S.* Enteritidis (ZM100) and Δ*virK* (ZM122), Δ*ybjX* (ZM123), and Δ*virKΔybjX* (ZM124) mutant strains (at logarithmic phase) to swim in 0.3% LB agar. As demonstrated in Fig. 2B, when compared to ZM100, all three mutants produced decreased swimming diameters (81.7% to 85.4% of ZM100; $p < 0.05$). The swimming competencies of the Δ*virK* and Δ*ybjX* mutants were partially restored when complemented with pWSK*VirK* and pWSK*YbjX*, respectively (Fig. 2B). Therefore, mutations in *virK* and *ybjX*, alone or in combination, alter the motility of *S.* Enteritidis.

3.4 S. Enteritidis ΔybjX mutant is sensitive to EDTA

To determine the sensitivity of wild type (ZM100), Δ*virK* (ZM122), Δ*ybjX* (ZM123), and Δ*virKΔybjX* (ZM124) to EDTA, each strain was seeded into LB agar and tested for its ability to grow in the presence of a filter disc containing 0.5M EDTA. The zones of inhibition were measured and shown as relative sensitivity (Fig. 4A). The Δ*ybjX* mutant was significantly more sensitive to 0.5M EDTA (9.1% more sensitive than wild type; $p<0.05$). However, the Δ*virK* and Δ*virKΔybjX* mutants were slightly more sensitive than the wild type to 0.5M EDTA, but not significantly (3.4% and 2.0% more sensitive than wild type, respectively). Growth in 0.5M EDTA was restored to the wild type phenotype when pWSK*virK* and pWSK*ybjX* were introduced into their respective

strains. This data indicate distinct roles for the two AMPR genes with regard to defense against EDTA, with *ybjX* having a greater role in this defense.

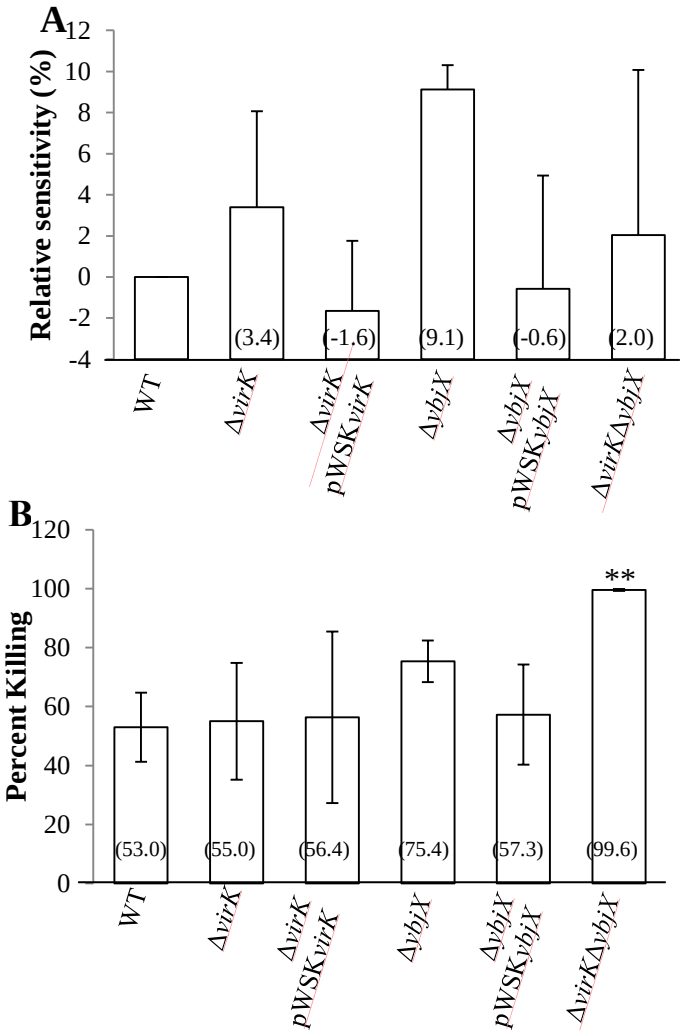

Figure 4. Sensitivity of AMPR mutants to EDTA and bile acid. Wild type *S.* Enteritidis (WT, ZM100), Δ*virK* (ZM122), Δ*ybjX* (ZM123), ΔvirKΔybjX (ZM124), Δ*virK* pWSK*VirK* (ZM122C), and Δ*ybjX* pWSK*YbjX* (ZM123C) were used to determine sensitivity to 0.5M EDTA and 1% DOC. **A.** Stationary phase *S.* Enteritidis were exposed to 0.5M EDTA and their zones of inhibition were measured and shown as the percent difference to WT. Mutant Δ*ybjX* had a significant increase in sensitivity to 0.5M EDTA. **B.** Ten-fold dilutions of log phase *S.* Enteritidis were grown on LB and LB containing 1% DOC; the percent of *S.* Enteritidis killing by 1% DOC compared to LB growth is shown. Mutant Δ*virK*Δ*ybjX* was significantly more sensitive to 1% DOC. Percent difference to WT **(A)** and percent killing **(B)** average values are shown in parentheses. Each assay was repeated for three independent trials.* denotes statistical significance ($p<0.05$) and ** denotes statistical significance ($p<0.01$).

3.5 S. Enteritidis AMPR mutants are sensitive to bile acids

To test if AMPR mutants have an increased sensitivity to natural detergents, we exposed the AMPR mutants to the bile acid deoxycholic acid *in vitro*. Equal amounts of ten-fold dilutions of log phase wild type (ZM100) and mutant strains, Δ*virK* (ZM122), Δ*ybjX* (ZM123), and Δ*virKΔybjX* (ZM124), were grown on LB agar and LB agar containing 1% deoxycholic acid (1% DOC). The percent killing of each strain is presented in Fig. 4B. While the wild type and Δ*virK* mutant demonstrated comparable susceptibilities to 1% DOC (53% and 55% killed, respectively), the Δ*ybjX* mutant was more susceptible (75.4% killed) than the wild type, however not significantly. The increased susceptibility to 1% DOC by the Δ*ybjX* mutant was restored to wild type sensitivity by introduction of pWSK*YbjX*. The double mutant, Δ*virKΔybjX*, was significantly more susceptible (99.6% killed; $p<0.05$) than any other strain to 1% DOC. These data indicate AMPR genes play distinct roles in the susceptibility of *S.* Enteritidis to 1% DOC, with the possibility that *ybjX* contributes more to this role than *virK*.

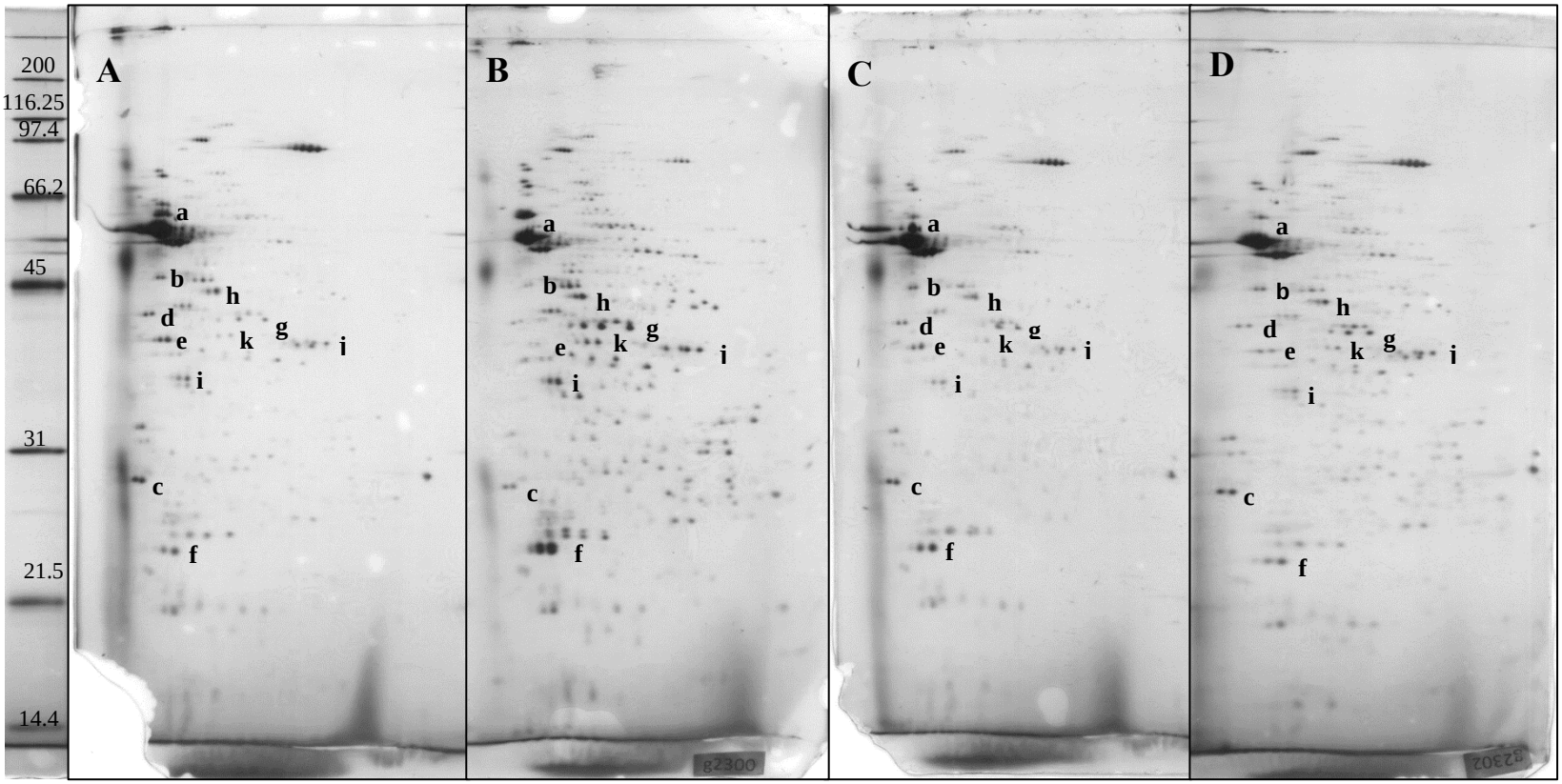

Figure 5. Differences in *in vitro* protein secretion between AMPR mutants. The proteins secreted into the culture supernatant from log phase wild type strain and AMPR mutants were extracted, separated using 2D SDS-PAGE, and analyzed densitormetrically. The Δ*virK*Δ*ybjX* mutant displayed the greatest differences in spot densities compared to the wild type strain. Representative 2D SDS-PAGE gels are shown with the protein ladder (kDa) to the far left and individual spots identitified **(a-f)** for later description in Table 4. Gels are as follows, **(A)** Wild type *S.* Enteritidis (ZM100), **(B)** Δ*virK*Δ*ybjX* (ZM124), **(C)** Δ*virK* (ZM122), and **(D)** Δ*ybjX* (ZM123). Cultures were run in triplicate and the experiment was repeated three times.

3.6 S. Enteritidis AMPR mutants have an altered profile of secreted proteins

The increased susceptibility of AMPR mutants to EDTA and bile acids suggested a weakened outer membrane. To evaluate the AMPR mutants' ability to appropriately secrete proteins, we extracted total proteins from the supernatant of log phase *S.* Enteritidis strains: wild type (ZM100), Δ*virK* (ZM122), Δ*ybjX* (ZM123), and Δ*virKΔybjX* (ZM124). We separated the secreted proteins using 2D SDS-PAGE and analyzed the difference in protein spot densities. The results of the 2D SDS-PAGE analysis showed differences in the protein quantities between the mutant strains and the wild type, with the most notable difference between wild type and the Δ*virKΔybjX* mutant (Fig. 5). We chose spots that differed at least 30% in density when the mutants were compared to the wild type. These spots (labeled in Fig. 5) were excised and sequenced by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and their identities shown in Table 4. The flagellar associated proteins (FliC, FliK, FlgD, and FlgE*)* and *Salmonella* T3SS-1 invasion protein (SipD) were most abundant in the wild type supernatant, decreased in the supernatant of Δ*virK* and Δ*ybjX* mutants and most strikingly decreased in the supernatant of the Δ*virKΔybjX* mutant. The FliC protein, phase-1 flagellin, was not detectable in any of the 2D SDS-PAGE gels of the Δ*ybjX* and Δ*virKΔybjX* mutants. Proteins that were found most abundantly in the supernatant of the Δ*virKΔybjX* mutant and with slightly higher abundance in the Δ*ybjX* mutant when compared to the wild type strain were associated with the cell wall or were localized within the cell: EF Tu, a cell membrane associated elongation factor Tu, OsmY, a periplasmic osmotically-inducible protein, MalE, a periplasmic maltose transporter

Table 4. *S.* Enteritidis proteins that vary in their *in vitro* secreted abundance between wild type and AMPR mutants.

Spot ID (Fig. 5)	Molecular Weight (kDa)	Gene symbol	Description	Cellular location	Protein coverage (%)	GenBank ID	Order of secreted protein abundance[A]
a	52	*fliC*	Phase-1 flagellin	Secreted	28.0	AAA27085.1	WT > Δv [B]
b	42	*fliK*	Flagellar hook-length control protein	Secreted	22.0	P26416.2	WT > Δv > Δy > ΔvΔy
c	24	*flgD*	Flagellar basal-body rod modification protein	Secreted	10.0	P0A1J0.1	WT > Δv = Δy > ΔvΔy
d	42	*flgE*	Flagellar hook protein	Secreted	40.0	P0A1J2.2	WT > Δv = Δy > ΔvΔy
e	37	*sipD*	T3SS-1 cell invasion protein	Secreted	34.0	Q56026.1	WT > Δv > Δy > ΔvΔy
f	21	*osmY*	Osmotically-inducible protein	Periplasm	27.0	P0AFH9.1	ΔvΔy > Δv = Δy > WT
g	43	*malE*	Maltose ABC transporter substrate binding protein	Periplasm	44.0	P19576.2	ΔvΔy > Δy > Δv > WT
h	43	*tuf*	Elongation factor Tu	membrane	5.3	A7ZSL4.1	ΔvΔy > Δv = Δy > WT
i	30	*tsf*	Elongation factor Ts	Cytosol	13.0	A8ALC0.2	ΔvΔy > Δv = Δy > WT
j	36	*gapA*	Glyceraldehyde -3-Phosphate dehydrogenase (GAPDH-A)	Cytosol	8.8	P0A9B4.2	ΔvΔy > Δy > Δv > WT
k	41	*glpQ*	Glycerophosphoryl diester phosphodiesterase	Periplasm	6.7	P09394.2	ΔvΔy > Δy > Δv > WT

[A] Order of protein abundance was determined densitometrically from three 2D SDS-PAGE gels for *S.* Enteritidis strains: wild type (WT), mutant Δ*virK* (Δv), mutant Δ*ybjX* (Δy), and mutant Δ*virK*Δ*ybjX* (ΔvΔy)

[B] FliC protein secretion was not detectable in the Δ*ybjX* (Δy) and Δ*virK*Δ*ybjX* (ΔvΔy) mutants with our methods.

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protein, GlpQ, a periplasmic glycerophosphoryl diester phosphodiesterase, EF Ts, a cytosolic elongation factor Ts, and GapA, a cytosolic GAPDH (Fig. 5 and Table 4).

3.7 S. Enteritidis AMPR mutants are more susceptible to antimicrobial peptides

To assess the contributions of *virK* and *ybjX* to antimicrobial peptide resistance in *S.* Enteritidis, a series of bacterial growth inhibition or killing assays were performed. Treatment of *S.* Enteritidis strains with polymyxin B, a potent antimicrobial peptide, caused in a 4-log reduction of the wild type S. Enteritidis and about 4.4-log reductions of the ΔvirK and ΔybjX mutants. Introduction of pWSKVirK and pWSKYbjX into the corresponding mutant strains complemented the increased sensitivity to polymyxin B (Fig. 6A). Subsequently, *S.* Enteritidis strains were treated with AvBD-6 which resulted in growth inhibition of mutant strains, *virK* (ZM112) and *ybjX* (ZM114), respectively, ranging from 24.1% to 30%, as compared to the 8.8% for the wild type *S.* Enteritidis (ZM100). The increased sensitivity of each mutant strain was complemented by low copy plasmids expressing the corresponding gene (Fig. 6B). These results indicate that both *virK* and *ybjX* contribute to the resistance of *S.* Enteritidis to avian beta-defensins, an important tool of the chicken innate immune system.

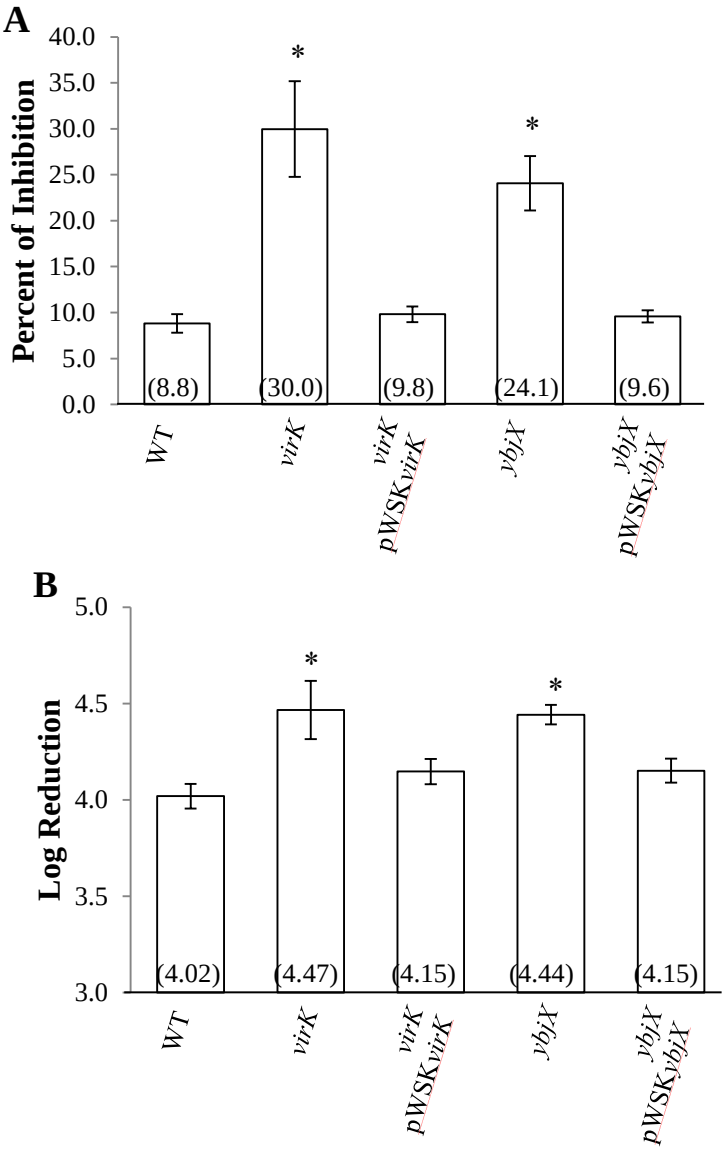

Figure 6. Sensitivity of AMPR mutants to antimicrobial peptides polymyxin B and AvBD-6. Wild type *S.* Enteritidis (WT, ZM100), *virK* (ZM112), *ybjX* (ZM114), *virK* pWSK*VirK* (ZM112C), and *ybjX* pWSK*YbjX* (ZM114C) were used to determine sensitivity to antimicrobial peptides **A.** *S.* Enteritidis grown in 10mM MgCl were exposed to a general, potent antimicrobial peptide, polymyxin B, for 1 h, then plated for recovery of viable *S.* Enteritidis and the results are shown as the log reduction. AMPR mutants, *virK* and *ybjX,* had an increase in reduction of recovered cells after exposure to polymyxin B. **B.** Log phase *S.* Enteritidis were tested for their sensitivity to host specific antimicrobial peptide, AvBD-6, using a micro-broth dilution method and the results are shown as percent of growth inhibition. The AMPR mutants, *virK* and *ybjX*, had increased sensitivity to AvBD-6. Each assay was repeated three times and the average values are shown in parentheses. * denotes significant differences ($p<0.05$).

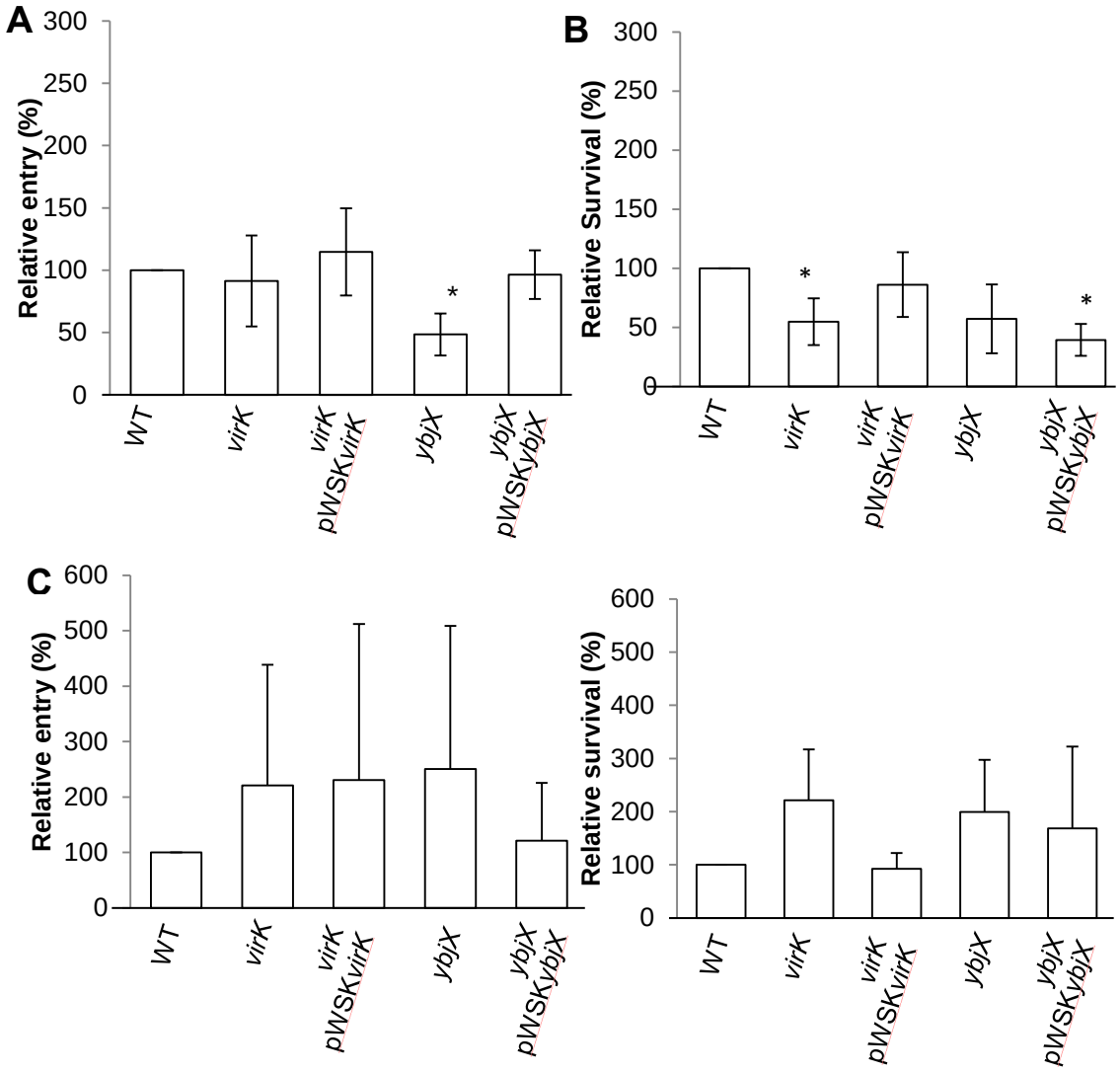

Figure 7. Relative entry and survival of AMPR mutants in chicken macrophage and reproductive epithelial cells. HD11 chicken macrophages and COEC were infected with stationary phase wild type *S.* Enteritidis (WT, ZM100), *virK* (ZM112), *ybjX* (ZM114), *virK* pWSK*VirK* (ZM112C), and *ybjX* pWSK*YbjX* (ZM114C) and a gentamicin protection assay was performed. *S.* Enteritidis recovered after 1 hpi were used to determine their ability to enter macrophages **(A)** or COEC **(C)**; the results are shown as a percent of the WT recovery. *S.* Enteritidis recovered after 16 hpi were used to determine their ability to survive inside macrophage **(B)** or COEC **(D)**; the results are shown as a percent of the WT recovery. Each assay was run in triplicate and repeated at least three separate times. The averages are displayed and * denotes statistical significance ($p<0.05$).

3.8 S. Enteritidis AMPR mutants have altered interactions with macrophage HD11 and COEC

To assess the interaction between the AMPR mutants and chicken cells, we used a gentamicin protection assay to test the contribution of *virK* and *ybjX* to the entry and survival of *S.* Enteritidis in avian macrophages (HD11) and primary chicken oviduct epithelial cells (COEC). To overcome motility defects, *S.* Enteritidis strains were centrifuged with the cells to synchronize infections. To assess the potential role of each gene in bacterial internalization and to avoid the SPI-1 T3SS-induced macrophage cell death, nonopsonized stationary phase bacteria were used to infect HD11 and COEC. The results are shown as a percentage of the wild type strain.

With respect to HD11 cells, *virK* was not involved in the entry of *S.* Enteritidis into these macrophages (Fig. 7A). In contrast, disruption of *ybjX* (ZM114) resulted in a significantly decreased entry of this strain into HD11 cells (45% of wild type; $p<0.05$). The defect demonstrated by the *ybjX* mutant was fully restored by introducing pWSKYbjX into the *ybjX* mutant (Fig. 7A). At 16 hpi, lower numbers of intracellular bacteria were recovered from HD11 cells infected with the *virK* and *ybjX* mutants than from cells infected with the wild type (Fig. 7B; *virK* $p<0.05$). The survival defect of mutant *virK* was partially complemented by the cloned gene. In contrast, introduction of pWSKYbjX into the mutant *ybjX* lead to reduced bacterial recovery from ZM114C-infected HD11 cells as compared to HD11 cells infected with the wild type or the *ybjX* mutant strain (Fig. 7B; $p<0.05$).

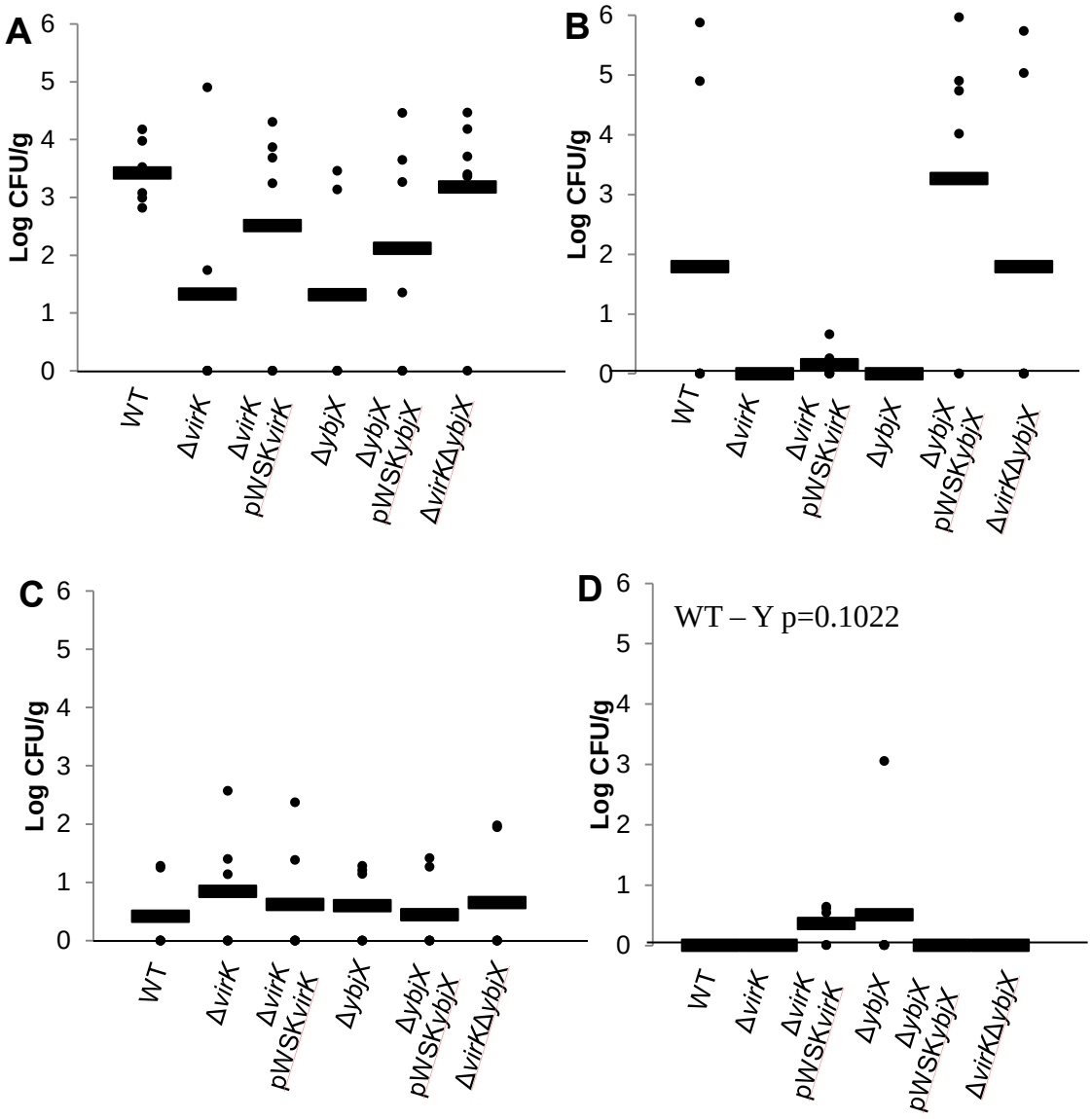

Figure 8. The bacterial loads of AMPR mutants in intestinal tissue. Wild type *S.* Enteritidis (WT, ZM100), Δ*virK* (ZM122), Δ*ybjX* (ZM123), ΔvirKΔybjX (ZM124), Δ*virK* pWSK*VirK* (ZM122C), and Δ*ybjX* pWSK*YbjX* (ZM123C) were used to orally infect 21-week old laying hens with 5 x 10^9 CFU. Hens were humanely euthanized at 5 and 10 dpi and their ileum and cecum were collected, homogenized, and plated to determine *S.* Enteritidis loads (log CFU/g): **(A)** ileum 5 dpi, **(B)** cecum 5dpi, **(C)** ileum 10dpi, and **(D)** cecum 10 dpi. There were differences in the *S.* Enteritidis loads recovered from 5 dpi tissue but not in the loads recovered at10 dpi tissue. Dots represent individual bird loads and bars represent the averages.

In COEC, the results showed the *ybjX* mutant was able to invade about twice as much as the wild type and the wild type phenotype was restored when pWSK*ybjX* was introduced into the *ybjX* mutant (250% and 121% of wild type, respectively; Fig. 7C). On the other hand, the *virK* mutant and its complement, *virK* pWSK*VirK*, were both able to invade about twice as much as the wild type (221% and 231% of wild type, respectively). It is worth noting the high variation in the ability of the mutants to invade COEC at 1 hpi and it was therefore hard to find significance at this time point. At 16 hpi, there was an increase in the recovery of the *virK* and *ybjX* mutants from inside COEC (221% and 199% of wild type, respectively; Fig. 7D). The recovery of *S.* Enteritidis returned to the wild type level with the introduction of pWSK*virK* into the *virK* mutant. Overall, in comparison to the wild type, AMPR mutants display altered interactions with chicken macrophages and oviduct epithelial cells *in vitro*.

3.9 S. Enteritidis AMPR mutants have a reduced ability to survive in the intestinal lumen and have reduced fecal shedding

To investigate the contribution of AMPR genes *virK* and *ybjX* to intestinal colonization and environmental spread during *S.* Enteritidis infection in chickens, we infected 21-week old laying hens with 5 x 10^9 CFU of wild type (ZM100), Δ*virK* (ZM122), Δ*ybjX* (ZM123), and Δ*virK*Δ*ybjX* (ZM124). At the same time point every day, fecal samples were collected from each hen. At 5 and 10 dpi, hens were humanly euthanized and ileum and cecum tissues were collected for laboratory analysis. In Fig. 8,

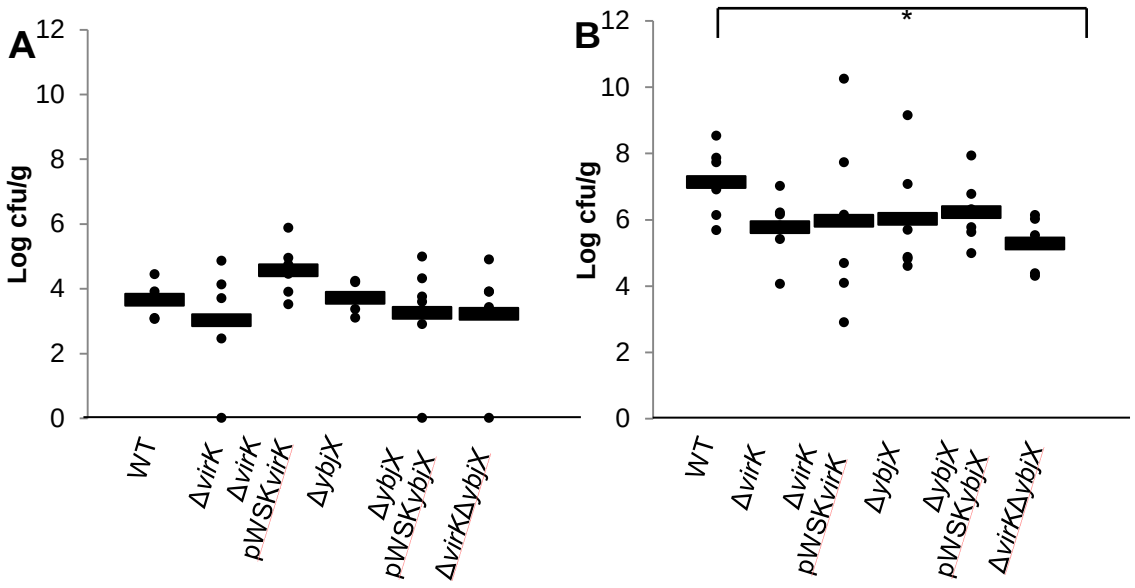

Figure 9. Survival of AMPR mutants in the ileum or cecum contents. Wild type *S.* Enteritidis (WT, ZM100), Δ*virK* (ZM122), Δ*ybjX* (ZM123), Δ*virKΔybjX* (ZM124), Δ*virK* pWSK*VirK* (ZM122C), and Δ*ybjX* pWSK*YbjX* (ZM123C) were used to orally infect 21-week old laying hens with 5 x 10^9 CFU. To dissect the differences seen in the early stage colonization of the intestinal tract, we plated the contents of the ileum and cecum for bacterial load (CFU/g): **(A)** ileum contents 5dpi, **(B)** cecum contents 5dpi. Significant differences were only found in the cecum contents (* denotes $p<0.05$). Dots represent individual bird loads and bars represent the averages.

the *S.* Enteritidis loads in each tissue are shown, and the fecal shedding loads are shown in Fig. 10.

At 5 dpi, the Δ*virK* and Δ*ybjX* mutants were less able to colonize the ileal and cecal epithelium, however not significantly (ileum, Δ*virK* p=0.0546 and Δ*ybjX* p=0.0556; cecum Δ*virK* p=0.1186 and Δ*ybjX* p=0.1186), (Fig. 8 A and B). In contrast, the Δ*virKΔybjX* mutant was able to colonize equally as well as the wild type in these tissues. Next, we cultured the ileum and cecum contents for their *S.* Enteritidis loads. As demonstrated in Fig. 9A, there was little difference in the *S.* Enteritidis loads between

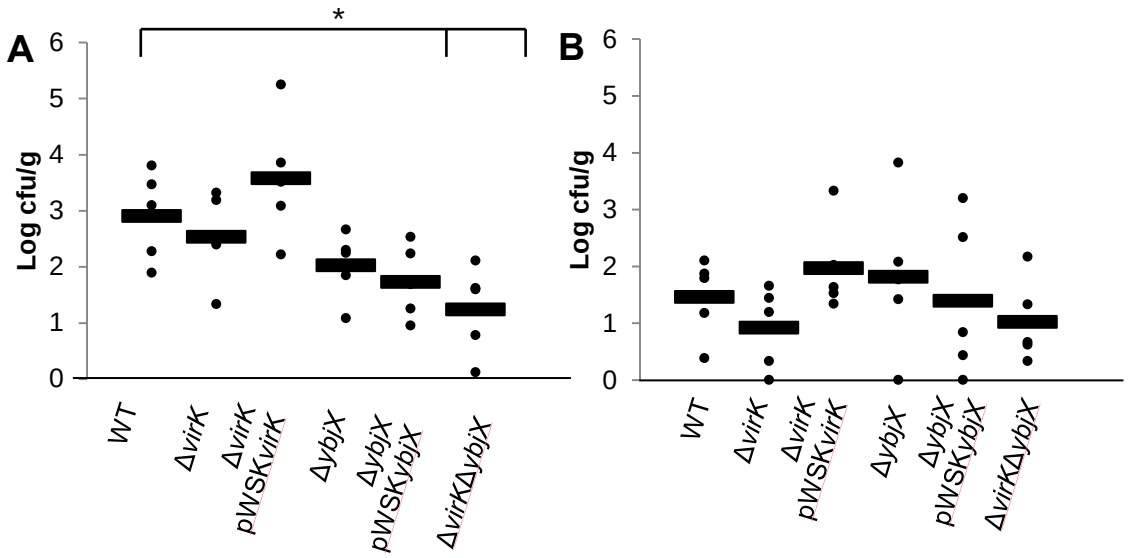

Figure 10. Fecal shedding of AMPR mutants during the early and late stages of infection. Wild type *S.* Enteritidis (WT, ZM100), Δ*virK* (ZM122), Δ*ybjX* (ZM123), Δ*virKΔybjX* (ZM124), Δ*virK* pWSK*VirK* (ZM122C), and Δ*ybjX* pWSK*YbjX* (ZM123C) were used to orally infect 21-week old laying hens with 5 x 10^9 CFU. At the same time point every day, a sample of feces was collected from each bird. One gram of feces was placed in BPW and ten-fold dilutions were plated to determine fecal loads (log CFU/g): (**A)** fecal loads from 1 to 5 dpi and **(B)** fecal loads from 6 to 10 dpi. Significant differences were seen in fecal loads collected 1 to 5 dpi (* denotes $p<0.05$). Each dot represents the average load of each strain per dpi and the bar represents the overall average from 5 days.

strains in the ileal contents. However, in the cecal contents, the Δ*virK* and Δ*ybjX* mutants had reduced loads and the Δ*virKΔybjX* mutant had the lowest *Salmonella* loads in their cecum content (Fig. 9B; Δ*virKΔybjX* $p<0.05$). Together, these data indicate that AMPR genes act distinctly in intestinal colonization through possibly antagonistic mechanisms. At 10 dpi, the Δ*ybjX* mutant was slightly more effective in colonizing the cecum than the

wild type (p=0.1022), but there were no significant differences between the strains' ability to colonize the intestinal tract (Fig. 8 C and D).

The inability to survive in cecal contents from 1 dpi to 5 dpi by the AMPR mutants coincides with reduced fecal shedding. As seen in Fig. 10, infections with AMPR mutants resulted in decreased fecal shedding of *S.* Enteritidis, with the lowest amount of fecal *Salmonella* from the hens infected with the Δ*virKΔybjX* mutant ($p<0.05$). From 6 dpi to 10 dpi, there were no statistically significant differences, but there was a numerical trend for the birds infected with AMPR mutants to have lower *S.* Enteritidis loads in their feces, except the Δ*ybjX* mutant.

3.10 S. Enteritidis Δ*ybjX* mutant is more effective in colonizing the spleen

To investigate the roles played by AMPR genes *virK* and *ybjX* during *S.* Enteritidis systemic infection in laying hens, we collected splenic tissue 5 dpi and 10 dpi. The *S.* Enteritidis loads from the spleen are shown in Fig. 11.

At 5 dpi, there were trends for the hens infected with the AMPR mutants to have low *Salmonella* bacterial counts in their spleen when compared to wild type *S.* Enteritidis (Fig 11A).The birds infected with the Δ*virKΔybjX* mutant had the lowest *Salmonella* loads (Fig. 11A; Δ*virKΔybjX* $p= 0.0668$). At 10 dpi, there was an increase in the variability of the splenic *Salmonella* loads compared to 5 dpi. In comparison to the wild type strain, the Δ*ybjX* mutant produced significantly higher splenic bacterial loads (Fig. 11B; $p<0.05$). In contrast, the Δ*virKΔybjX* mutant had the lowest amount of *Salmonella* recovered from the spleen at this time point. When the cloned gene for *virK*

and *ybjX* were introduced into their respective strains, wild type trends were restored, with the exception of the Δ*ybjX* pWSK*YbjX* strain at 5 dpi.

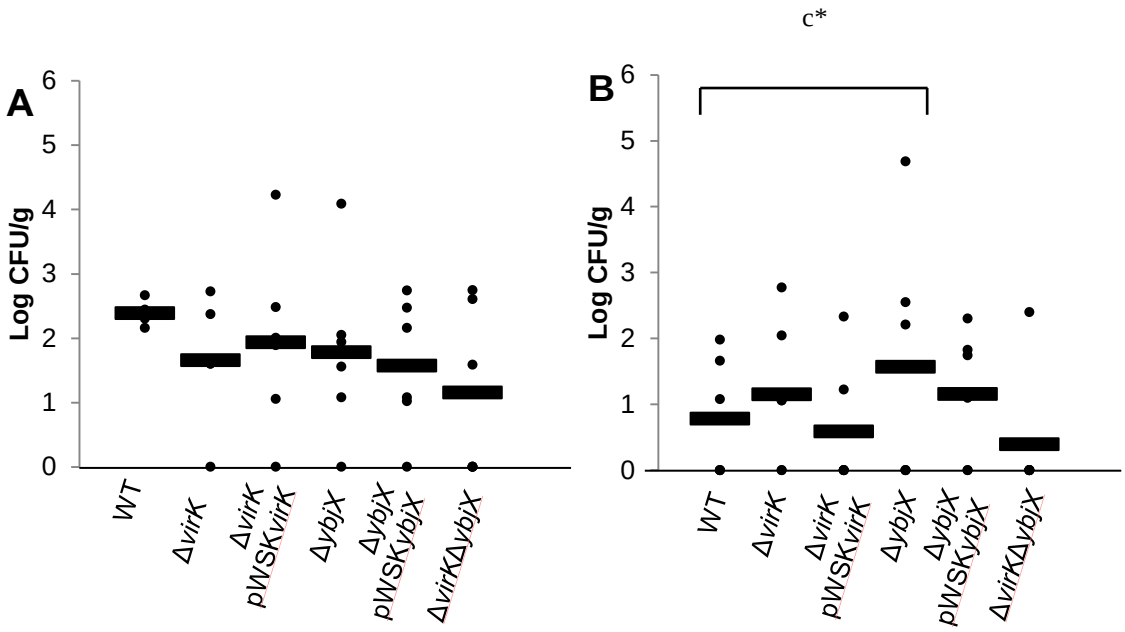

Figure 11. The bacterial loads of AMPR mutants in splenic tissue. Wild type *S.* Enteritidis (WT, ZM100), Δ*virK* (ZM122), Δ*ybjX* (ZM123), Δ*virKΔybjX* (ZM124), Δ*virK* pWSK*VirK* (ZM122C), and Δ*ybjX* pWSK*YbjX* (ZM123C) were used to orally infect 21-week old laying hens with 5 x 10^9 CFU. Hens were humanely euthanized at 5 dpi and 10 dpi and their spleen was collected, homogenized, and plated to determine *S.* Enteritidis loads (log CFU/g or log CFU/ovary): **(A)** spleen 5dpi **(B)** spleen 10 dpi. Dots represent bacterial load in individual birds, bars represent the average bacterial load in individual groups, and $*^{C}$ denotes statistical significant difference in the amount of *Salmonella* in the tissue that was colonized ($p<0.05$)

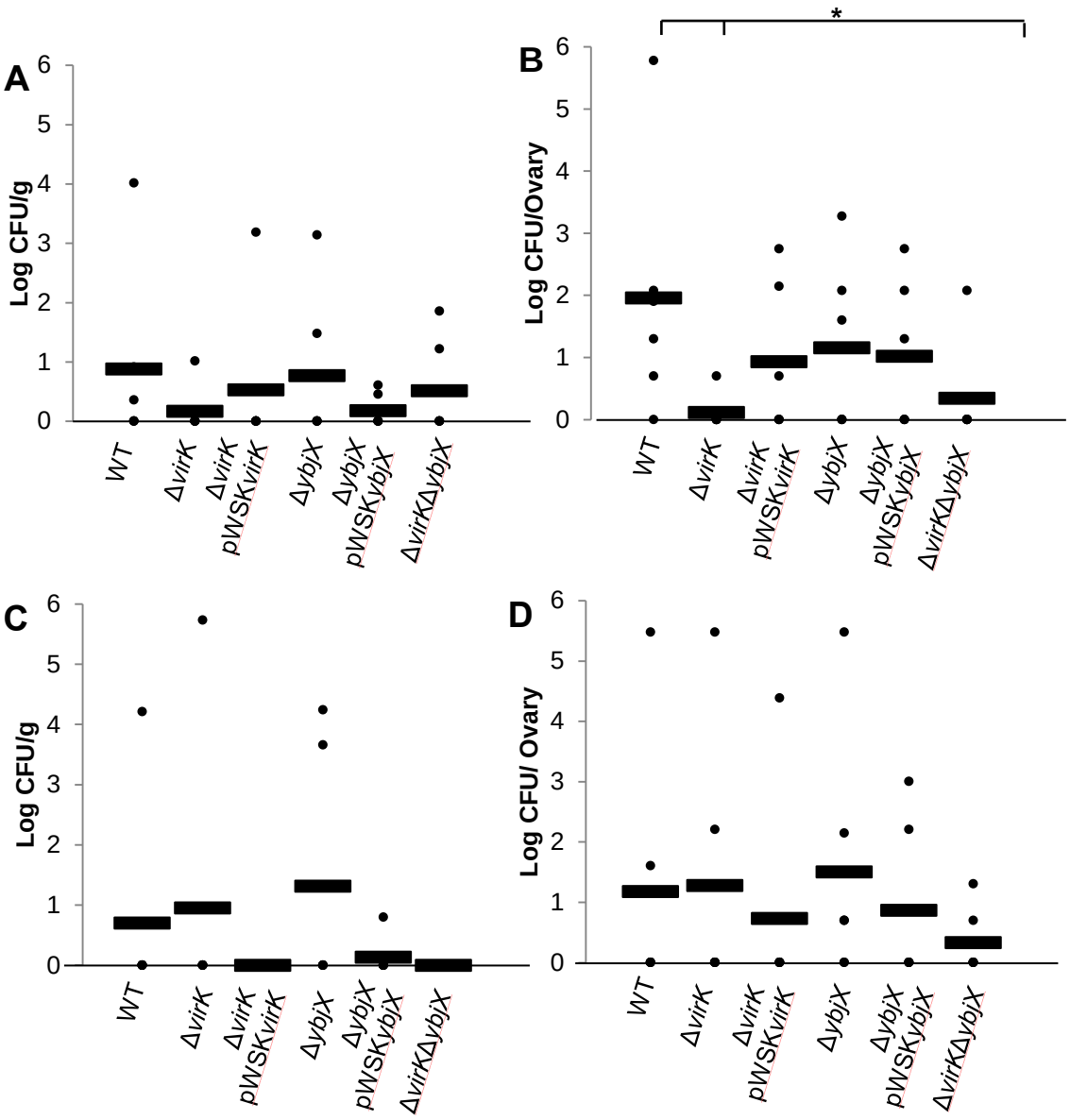

Figure 12. The bacterial loads of AMPR mutants in reproductive tissue. Wild type *S.* Enteritidis (WT, ZM100), Δ*virK* (ZM122), Δ*ybjX* (ZM123), ΔvirKΔybjX (ZM124), Δ*virK* pWSK*VirK* (ZM122C), and Δ*ybjX* pWSK*YbjX* (ZM123C) were used to orally infect 21-week old laying hens with 5 x 10^9 CFU. Hens were humanely euthanized at 5 and 10 dpi and their oviduct (isthmus) and ovary were collected, homogenized, and plated to determine *S.* Enteritidis loads (log CFU/g or log CFU/ovary): (A) oviduct (isthmus) 5 dpi (B) ovary 5 dpi (C) oviduct (isthmus) 10 dpi and (D) ovary 10 dpi. Dots represent bacterial load in individual birds, bars represent the average bacterial load in individual groups, and * denotes statistical significance ($p<0.05$).

3.11 *S.* Enteritidis AMPR genes are needed to colonize the reproductive tract and contaminate eggs

To investigate the roles played by AMPR genes *virK* and *ybjX* during *S.* Enteritidis colonization of the reproductive tract of laying hens and ultimately the contamination of eggs, we collected eggs at the same time every day and collected the oviduct (isthmus) and ovary tissue at 5 dpi and 10 dpi. The *S.* Enteritidis load in each tissue is shown in Fig. 12 and the percent of contaminated eggs for each strain is shown in Table 5.

At 5 dpi, AMPR mutants varied in their ability to colonize reproductive tissue. In the oviduct, the Δ*virK* mutant had lower bacterial loads than the wild type (Fig. 12A). In the ovary, there was a significantly reduced amount of *S.* Enteritidis recovered from birds infected with Δ*virK* and Δ*virK*Δ*ybjX* mutants (Fig. 12B; $p<0.05$). In contrast, the Δ*ybjX* mutant did not show a defect in reproductive tissue colonization. When observing the number of ovaries from which we cultured each strain 5 dpi, the wild type strain infected the most ovaries (83.3%), the Δ*ybjX* mutant infected less (50.0%), and the Δ*virK* and Δ*virK*Δ*ybjX* mutants infected the least number of ovaries (16.7%; Table 5). When comparing these data to the percent of contaminated eggs, we noticed a large number of infected eggs from hens inoculated with the Δ*virK* mutant at 1 dpi (50%), but then a steady decline of egg deposition to 3 dpi (16.7%) compared to the wild type, which contaminated eggs increasingly to 4 dpi (20% up to 33.3%; Table 5). The complemented Δ*virK* strain (ZM122C) was able to restore the wild type S. Enteritidis phenotype. The Δ*ybjX* mutant was more effective than the wild type strain in egg

Table 5. *S.* Enteritidis deposition in eggs[A] and colonization in ovaries[B].

	Number of Positive/Total (% Positive) Eggs												Number of Positive/Total Ovaries	
Strain\Day	1	2	3	4	5	6	7	8	9	10	Average % Positive	Total (%)	5	10
WT	1/5 (20.0)	1/5 (20.0)	1/5 (20.0)	1/3 (33.3)	0/6 (0.0)	0/2 (0.0)	0/2 (0.0)	0/1 (0.0)	0/2 (0.0)	0/4 (0.0)	(9.33)	4/35 (11.4)	5/6 (83.3)	2/6 (33.3)
ΔvirK	4/8 (50.0)	1/7 (14.3)	1/6 (16.7)	0/4 (0.0)	0/6 (0.0)	0/4 (0.0)	0/3 (0.0)	0/4 (0.0)	0/4 (0.0)	0/6 (0.0)	(8.1)	6/52 (11.5)	1/6 (16.7)	2/6 (33.3)
ΔvirK pWSK*virK*	1/6 (16.7)	2/8 (25.0)	0/3 (0.0)	2/5 (40.0)	2/8 (25.0)	0/6 (0.0)	0/3 (0.0)	0/3 (0.0)	0/2 (0.0)	0/7 (0.0)	(10.7)	7/51 (13.7)	3/6 (50.0)	1/6 (16.7)
ΔybjX	2/6 (33.3)	1/7 (14.3)	1/2 (50.0)	2/4 (50.0)	2/4 (50.0)	1/4 (25.0)	1/1 (100)	0/2 (0.0)	1/3 (33.3)	0/4 (0.0)	(35.5)*	11/37 (29.7)	3/6 (50.0)	4/6 (66.7)
ΔybjX pWSKybjX	2/3 (66.7)	0/2 (0.0)	0/2 (0.0)	0/3 (0.0)	0/2 (0.0)	0/4 (0.0)	0/2 (0.0)	0/1 (0.0)	0/2 (0.0)	0/4 (0.0)	(6.7)	2/25 (8.0)	3/6 (50.0)	2/6 (33.3)
ΔvirKΔybjX	2/5 (40.0)	1/3 (33.3)	2/3 (66.7)	1/2 (50.0)	0/3 (0.0)	0/2 (0.0)	0/1 (0.0)	0/1 (0.0)	0/1 (0.0)	0/2 (0.0)	(19.0)	6/23 (26.1)	1/6 (16.7)	2/6 (33.3)

[A] Eggs were collected at the same time daily during the infection challenge of 21-week old laying hens with 5 x 10^9 CFU of *S.* Enteritidis strains. Grey boxes highlight the positive eggs as the fraction of total eggs collected each dpi and the percentage shown in parentheses.
[B] The number of hens with positive ovary colonization shown as the fraction of the total hens tested at each tissue collection time point (5 dpi and 10 dpi respectively) and the percentage shown in parentheses.
* Denotes significant difference to WT in average positive eggs/ dpi (%) and in the amount of positive dpi (grey boxes) ($p<0.05$).

deposition as indicated by the higher percentage of contaminated eggs and longer period of contamination (up to 9 dpi). The Δ*virKΔybjX* mutant had steady egg deposition to 4 dpi which was similar to the wild type phenotype. While the complemented Δ*virK* strain (ZM122C) was more able to colonize ovaries and eggs, the complemented Δ*ybjX* strain (ZM123C) was less virulent than the mutant in terms of reproductive tissue colonization and egg deposition (Fig. 12 and Table 5). The data suggests that AMPR genes play distinct roles in reproductive tract colonization and defense against innate immunity with *virK* contributing the most in reproductive tract colonization and egg deposition during the early stages of infection.

At 10dpi, fewer *S.* Enteritidis organisms were recovered and there was an increase in the variability of the strains to colonize reproductive tissue compared to 5 dpi tissue. The Δ*virK* and Δ*ybjX* mutants were able to colonize the oviduct and ovary equally as well as the wild type, with the Δ*ybjX* mutant having slightly higher loads in all reproductive tissue at this time point (Fig. 12 C and D). On inspection of the number of colonized ovaries at 10dpi, we observed the Δ*ybjX* mutant infected the most ovaries (66.7%) while the Δ*virK* and Δ*virKΔybjX* mutants infected as many as the wild type (33.3%; Table 5). When looking at egg deposition from 6 dpi to 10 dpi, the Δ*ybjX* mutant was the only strain isolated from egg contents (Table 5); this may be associated with the higher bacterial loads found within the oviduct and ovary from hens infected with this strain.

4. DISCUSSION AND CONCLUSIONS

The main vehicle of *S.* Enteritidis infection in humans is thought to be contaminated eggs and poultry products [3]. The prevalence of *S.* Enteritidis associated infections has declined since 1999, but outbreaks associated with this organism seem to persist in our society [4,5]. Compared to other serovars, persistent outbreaks have been hypothesized to occur because *S.* Enteritidis is more suitable to persistently colonize the reproductive tract of laying hens without inducing overt clinical symptoms [31]. While many studies have demonstrated that *S.* Enteritidis exploits inherent differences to colonize the reproductive tract and contaminate eggs better than other serovars, the exact mechanisms for this action have yet to be discovered [28, 31, 33, 52].

Many experiments with *S.* Enteritidis focus on the mechanisms of the T3SS-1 and T3SS-2 during invasion and intracellular replication within various chicken tissues [17, 53-55]. Recent studies have focused on mechanisms that are required by *S.* Enteritidis to survive in the stressful conditions of the chicken such as those present in egg white. These studies have identified mutations in *rpoS*, SPI-14 genes, and *ksgA* that cause specific attenuation in *S.* Enteritidis virulence and specific attenuation in chicken liver invasion and macrophage survival [56, 57]. In the present study, we used a selective capture of transcribed genes (SCOTS) assay to identify the genes over-expressed by *S.* Enteritidis upon entry into chicken macrophages (HD11) and chicken oviduct epithelial cells (COEC) and characterized those genes that were identified as antimicrobial peptide resistance genes (AMPR genes).

Salmonella utilizes macrophages as a transport vessel to invade systemic sites within the host [6]. Once *S.* Enteritidis invades the reproductive tract, successful egg contamination by *S.* Enteritidis most likely happens during egg development with predominant *S.* Enteritidis colonization in the isthmus of the oviduct [28, 58]. Therefore in our study, selection of chicken macrophage HD11 cells and COEC was essential to identify those genes employed by *S.* Enteritidis during successful and persistent reproductive tract colonization. The genes found to be over-expressed in HD11 and COEC consisted of a cohort of genes involved in stress response, transport, cell wall and DNA modification, fimbrial, AMPR, and virulence. The identification of SPI-1 genes 1 hpi and SPI-2 gene 4 hpi validated the authenticity of the SCOTS experiment as these genes are known to be involved in invasion and intracellular replication, respectively [18, 23, 26]. The over-expression of SPI-2, SPI-5, and AMPR genes were confirmed by real-time PCR, indicating the utilization of these genes for replication and survival within these chicken cells. SPI-2 genes and the SPI-5 gene, *pipB,* have been studied for their effects during *S.* Enteritidis infection in hens, but there is a lack of knowledge of the role AMPR genes *virK* and *ybjX* play during *S.* Enteritidis infection in chickens [17, 54, 55, 59].

AMPR genes *virK* and *ybjX* discovered in the SCOTS experiment are a part of the PhoP/PhoQ regulon, which consists of over 40 genes speculated to modulate the bacterial outer membrane to contribute to antimicrobial resistance, virulence, and survival in low Mg^{+2} conditions [10-12, 15, 16]. In *Shigella flexneri*, *virK* is hypothesized to modulate the outer membrane to alter the interaction between IcsP (an

actin modulating protein) and lipid A [16]. In *S.* Typhimurium, *ybjX* was found as a suppressor mutation (initially termed *somA*) to a mutation in the lipid A assembly protein MsbB, functionally linking *ybjX* to outer membrane modification [60]. The outer membrane serves several functions for the bacteria including stabilization of various functions, such as movement and secretion, and for defense against the host's killing tactics. Mutations that affect outer membrane stability make the bacteria sensitive to detergents, to which *Salmonella* is naturally resistant [13, 46]. We have shown that AMPR genes in *S.* Enteritidis contribute to outer membrane stability for resistance to EDTA and bile acid deoxycholate (Fig 4). EDTA is capable of chelating the divalent cations Mg^{+2} and Ca^{+2} that link the outer membrane lipopolysaccharide (LPS) molecules. To overcome the reduction in connective cations caused by EDTA, *Salmonella enterica* employs membrane stabilizing mechanisms to maintain a strong barrier. For example, a *S.* Typhimurium *msbB* mutant lacks the ability to add myristic acid to the lipid A portion of LPS. The inability to add more stabilizing fatty acids to anchor LPS molecules into the outer membrane results in an increased susceptibility to EDTA [60]. Mutations that abolish the synthesis of lipid A, and thus LPS, are lethal to enteric bacteria because LPS makes up a majority of the outer membrane and is responsible for most of its characteristics [61].

In *S.* Typhimurium, a mutation in *msbB* caused formation of elongated cells and a mutation in *tatB* or *tatC* (twin arginine transport proteins required for transporting outer membrane components) caused long, aggregate filaments to form [46, 60]. Although a mutation in *somA* (*ybjX*) suppressed many phenotypes associated with the

msbB mutant, the phenotypes related to a single mutation in *somA* (*ybjX*) were not characterized [60]. In the current study, we observed an increase in cell length and filamentous formations for the Δ*ybjX* and Δ*virK*Δ*ybjX* mutants (Fig. 2 and 3). Therefore, the similar morphology seen with our mutants and the mutants in previous studies that altered an outer membrane component collectively suggest that AMPR genes in *S.* Enteritidis play a role in outer membrane modulation.

Flagella contribute to bacterial virulence and proper flagellum formation and motorization requires a stable outer membrane [45, 62, 63]. This study shows that mutations in AMPR genes *virK* and *ybjX* have an impact on *S.* Enteritidis motility. It is likely that these two genes disrupt flagellar function by the same mechanism because the double mutant (Δ*virKΔybjX*) displayed similar motility defects as the individual mutants (Δ*virK* and Δ*ybjX*) (Fig. 2B). In *S.* Typhimurium, a mutation in *msbB* (*waaN*) resulted in an inability to secrete *Salmonella* effector proteins [48, 60]. The current study shows that mutations in AMPR genes affect the ability to secrete proteins required for flagella function, indicating an unstable membrane (Fig. 5, Table 4). FliC, FliK, FlgD, FlgE proteins are secreted through a secretion system similar to the type three and the type five secretion systems [63, 64]. The inability to secrete these flagellar proteins may explain the decreased motility seen in the three AMPR mutants [65]. The decreased secretion of SipD, a T3SS-1 invasion protein, in the AMPR mutants may alter the ability for these cells to invade host tissue [18]. Mutations in AMPR genes also caused an increase of proteins in the supernatant that are naturally found in the cell membrane (Tuf [66]), periplasm (OsmY, MalE, and GlpQ [67-69]), or in the cytosol (Tsf and GapA [70,

71]). The increased presence of these proteins in the supernatant of the Δ*virK*Δ*ybjX* and Δ*ybjX* indicate a leaky membrane or membrane shearing.

A study in *S.* Typhimurium has shown that these AMPR genes, through direct or indirect modification of LPS, contribute to resistance to antimicrobial peptide polymyxin B [11]. We have shown in this study that *virK* and *ybjX* are not only involved in resistance to polymyxin B, but that they are also involved in resistance to AvBD-6, an antimicrobial peptide crucial in the chicken innate immune system (Fig. 6). Therefore, it is clear that these AMPR genes are altering the stability of the outer membrane of *S.* Enteritidis which in turn affects the ability of the organism to coordinate virulence functions and defend itself against innate antimicrobial peptides.

PhoP/PhoQ-regulated genes have been shown to aid in virulence and play a role in late stages of *S.* Typhimurium infection in mice [10, 11, 15]. The hypothesis behind this phenomenon is that genes in the PhoP/PhoQ regulon, known to be up-regulated within macrophages, alter the outer membrane structure or composition to defend itself against the various host killing factors within these phagocytes [10, 14].We have seen that a mutation in *ybjX* is associated with decreased macrophage entry; either due to a suboptimal interaction with the macrophage or due to the inability of macrophages to phagocytize the larger bacteria (Fig 2, 3 and 7). Once inside macrophages, an inability to survive and replicate can hinder *Salmonella* from systemic spread. We have observed a survival defect of AMPR mutants inside macrophages and a slight defect in spleen colonization 5 dpi (Fig. 7 and 11). Our data are in agreement with findings in a previous study with *S.* Typhimurium in mice that *virK* plays a larger role in systemic spread than

ybjX [11]. This study also found that a double mutant (*virK ybjX)* was not more attenuated in systemic spread than either single mutant, indicating the two genes operate virulence with similar mechanisms [11]. In the present study, infection with the double mutant, when compared to the single mutants, resulted in the lowest level of spleen colonization (Fig. 11). The data collectively suggest that these genes have different roles in systemic spread of *S.* Enteritidis in chickens but not *S.* Typhimurium in mice. Furthermore, at 10 dpi, we observed that the Δ*ybjX* mutant survived better than the wild type in the spleen, although the Δ*virKΔybjX* mutant was the least recovered from the spleen at this time point. This indicates that conjointly, AMPR genes play a role in maintaining systemic spread but not individually. This is contrasting with the observation that both these AMPR genes play a role in late stages of infection in *S.* Typhimurium in mice [11]. One explanation for this is the possibility that stress induced mechanisms, such as genes in the PhoP/PhoQ regulon, are alternatively utilized by different serovars to modulate their outer membranes to cope with their preferential hosts.

To determine the contribution of AMRP genes to intestinal colonization, we analyzed the intestinal and fecal bacterial loads. In early stages of infection, the double mutant (Δ*virKΔybjX*) was excreted the least (Fig. 10) which coincided with its inability to survive in cecum contents (Fig. 9) and its inability to withstand the bile acid detergent deoxycholic acid (Fig. 4). Although *virK* and *ybjX* are needed individually for ileum and cecal colonization, these two genes operate in counteracting mechanisms as evidenced by the fact that the double mutant (Δ*virKΔybjX*) colonized intestinal tissue as well as the

wild type (Fig. 8). In *S.* Typhi, the outer core of LPS is required for entry into intestinal epithelial cells, which may explain how the unstable membrane of our AMPR mutants impacts intestinal invasion [20]. At 10dpi, the AMPR mutants colonized the intestine as well as the wild type and the mutants were shed in the feces similarly to the wild type. This indicates these genes are not utilized during later stages of infection in the intestine. In conclusion, AMPR genes display individual roles in bacterial defense against host innate tactics as well as invasion of intestinal tissue to spread through shedding during the early stages of infection.

S. Enteritidis is known to preferentially colonize the reproductive tract of laying hens and contaminate eggs without inducing overt clinical signs [31, 32]. Furthermore, *S.* Enteritidis strains recovered from the field have a higher degree of heterogeneity, especially in the glycosylation in the O-chain of the LPS, compared to *S.* Typhimurium [29, 30]. This heterogeneity may be caused by alternative implementation of outer membrane modification stress mechanisms in the various host environments for *S.* Enteritidis' defense [31, 72]. We have observed heterogeneity and alternative utilization with our AMPR genes in *S.* Enteritidis infection in hens. While both *virK* and *ybjX* are important in intestinal colonization and fecal shedding in the early stages of infection (Fig. 8 and 10), we have observed a different phenotype of our AMPR mutants in the reproductive tract. A mutation in *virK* renders *S.* Enteritidis from colonizing the oviduct and ovary even more so than a mutation in both *virK* and *ybjX*. Furthermore, our Δ*ybjX* mutant was able to survive in oviduct and ovary better than all the other strains at 10 dpi as evident by the ability to contaminate the most ovaries (Fig. 12 and Table 5). Also, the

Δ*ybjX* mutant contaminated the most eggs up to 9 dpi, while the other strains did not produce egg deposition past 5 dpi (Fig. 12, Table 5). It has been previously shown that *S.* Enteritidis mutant (*wzz*) lacking high molecular mass LPS (HMM-LPS) was more effective than the wild type strain in reproductive tract colonization and egg deposition [73]. It was suggested that HMM-LPS favorably allows *S.* Enteritidis to silently colonize the chicken host [73]. Our data suggest *virK* is required for early reproductive tract colonization and *ybjX* negatively controls egg deposition, possibly through modulation of the bacterial outer membrane and/or interactions with chicken immune responses. Apparently, the utilization of *S.* Enteritidis AMPR genes varies within the different host environments to combat the organ-specific defenses deployed by the chicken.

S. Enteritidis and *S.* Typhimurium are anywhere from 6.4% to 9.6% variant in their genes with the most obvious difference being the chemical structure of their outer membrane, one of the key components to the interaction between *Salmonella* and its host [34, 35]. Many scientists have argued that the main difference between *S.* Typhimurium and S. Enteritidis is the way that they each use stress-induced mechanisms, including outer membrane modulation, to survive in the chicken host; these differences are responsible for the differences seen in epidemics associated with contaminated poultry products. [6, 29, 31, 72]. Silent colonization of *S.* Enteritidis in chicken requires intrinsic abilities to defend against the innate immune system without inducing overt inflammation and damage [32]. This study confirms that modulation of the *S.* Enteritidis outer membrane by AMPR genes *virK* and *ybjX* not only aids in resistance against innate

antimicrobial peptides and detergents, but by modulating the outer membrane these genes are also involved in invasion and replication in chicken tissue and in survival in egg contents. Furthermore, the effects of AMPR genes seen in *S.* Enteritidis do not coordinate with the effects in an infection of mice with *S.* Typhimurium. Future experiments will be aimed at elucidating the exact mechanistic actions of outer membrane modulation by AMPR genes and looking at how these outer membrane modulations reshape the interaction with the chicken immune system to aid in silent colonization.

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APPENDIX

Table 6. Overview of *in vitro* characterization of *S.* Enteritidis AMPR mutants.

	Phenotypic characteristic				
Experiment	Wild type	*virk* mutant	*ybjX* mutant	*virK ybjX* mutant	AMPR contribution to phenotype[A]
Reverse transciptase real-time PCR	N/A	COEC 4 h, HD11 4 h	COEC 4 h, HD11 4 h	N/A	
Morphology	rod shape	rod shape	filaments	Long filaments	*virK* < *ybjX*
Length 4 h (um)	2.4	2.6	3.2*	4.3*	*virK* < *ybjX*
Length 16 h (um)	1.9	1.9	2.5*	2.8*	
Motility (Relative %)	100%	81.7%*	85.4%*	82.1%*	*virK*= *ybjX*
EDTA sensitivity (Relative %)	0%	3.40%	9.1%*	2.04%	*virK* < *ybjX*
1% DOC sensitivity (% killed)	53.00%	55.00%	75.38%	99.61%*	*virK* < *ybjX*
Polymyxin B sensitivity (Log reduction)	4.02	4.47*	4.44*	N/A	*virK*= *ybjX*
AvBD-6 Sensitivity (% inhibition)	8.80%	29.97%*	24.07%*	N/A	*virK* = *ybjX*
HD11 entry (Relative %)	100%	91.38%	48.47%*	N/A	*ybjX*
HD11 survival (Relative %)	100%	54.82%*	57.18%	N/A	*virK* > *ybjX*
COEC entry (Relative %)	100%	220.95%	250.42%	N/A	*virK* = *ybjX*
COEC survival (Relative %)	100%	221.44%	199.18%	N/A	*virK* = *ybjX*

[A] Contribution of the AMPR gene based on the individual and combined effects of the mutants: < or >, indicate one gene contributes more to the phenotype than the other, = indicates they are similarly involved in the phenotype, possibly using similar mechanisms, and if only one gene is listed it indicates the other AMPR gene was not found to contribute to the phenotype.

* Denotes statistical significant difference to the wild type ($p<0.05$).

Table 7. Overview of *S.* Enteritidis AMPR mutants during an infection challenge of 21-week old laying hens.

		Phenotype Observed[A]				
	Experiment	Wild type	*virk* mutant	*ybjX* mutant	*virK ybjX* mutant	AMPR contribution to phenotype[B]
5 dpi	Ileum colonization	3.42	1.32	1.32	3.18	*virK* & *ybjX*
	Ileum content survival	3.66	3.03	3.26	3.23	neither
	Cecum colonization	1.79	0.00	0.00	1.79	*virK* & *ybjX*
	Cecum content survival	7.14	5.77	6.03	5.29*	*virK* & *ybjX*
	Fecal shedding 1 dpi - 5 dpi	2.90	2.54	2.03	1.24*	*virK* & *ybjX*
	Spleen colonization	2.39	1.66	1.78	1.16	*virK = ybjX*
10 dpi	Oviduct colonization	0.88	0.17	0.77	0.51	*virK > ybjX*
	Ovary colonization	1.96	0.12*	1.16	0.35*	*virK > ybjX*
	Ileum colonization	0.42	0.85	0.60	0.65	*virK = ybjX*
	Cecum colonization	0.00	0.00	0.51	0.00	*ybjX*
	Fecal shedding 6 dpi - 10 dpi	1.47	0.93	1.82	1.02	*virK* & *ybjX*
	Spleen colonization	0.79	1.15	1.57 *[C]	0.40	*virK* & *ybjX*
	Oviduct colonization	0.70	0.95	1.31	0.00	*virK* & *ybjX*
	Ovary colonization	1.18	1.28	1.50	0.33	*virK* & *ybjX*
	Egg deposition	Increasing to 4 dpi	Decreasing to 3 dpi	Interval to 9 dpi	Steady up to 4 dpi	virK < ybjX

[B] Phenotypic values are listed as the average *Salmonella* load per experiment for each AMPR mutant (values are log CFU/g, except ovary values are log CFU/ovary).

[A] Contribution of the AMPR gene based on the individual and combined effects of the mutants: "< or >" indicate one gene contributes more to the phenotype than the other, "=" indicates they are similarly involved in the phenotype, possibly using similar mechanisms, and "&" indicates both AMPR genes contribute differently to the phenotype in either subtracting and/or adding mechanisms.

* Denotes statistical significant difference to the wild type and *[C] denotes statistical significant difference to the wild type between the amount of *Salmonella* in those tissue that were colonized ($p<0.05$).