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Salmonella enterica Serovar Enteritidis Antimicrobial Peptide Resistance Genes Aid in Defense against Chicken Innate Immunity, Fecal Shedding, and Egg Deposition

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Salmonella enterica **serovar Enteritidis (*S.* Enteritidis) is a major etiologic agent of nontyphoid salmonellosis 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 overexpressed in the HD11 chicken macrophage cell line 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 (AMPR) genes, *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. In the intestinal tract, AMPR genes were involved in early intestinal colonization and fecal shedding. In the reproductive tract, *virK* was required in early colonization whereas a deletion of *ybjX* caused prolonged ovary colonization and egg deposition. Data from the present study indicate that AMPR genes are differentially utilized in various host environments, which may ultimately assist *S.* Enteritidis in persistent and silent colonization of chickens.**

Salmonella enterica serovars are important zoonotic pathogens that cause 1.028 million nontyphoidal salmonellosis cases with approximately 400 deaths annually in the United States (1). Since 1994, *Salmonella enterica* serovar Enteritidis has been the predominate serovar isolated from nontyphoidal salmonellosis cases in the United States, with a majority of these cases associated with the consumption of contaminated poultry products (2). Since the 1990s, eggs have been a significant source of infection and chicken products have been identified as a significant risk factor for human 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). However, even with these increased control measures in place, *S.* Enteritidis continues to be a major health risk associated with significant economic losses. For example, in August 2010, the United States had to recall 500 million eggs during an *S.* Enteritidis outbreak in Iowa that spread to 11 states and caused over 1,939 illnesses (5).

Egg contamination can be a result of horizontal transmission, of transmission from the environment after the egg is laid, or of vertical contamination from *S.* Enteritidis colonizing the reproductive tract (2). *Salmonella* can spread in a hen house very quickly through the fecal-oral route. After ingestion, the mildly acidic crop primes *Salmonella* genes for the acidic environment faced in the intestinal tract, where the organism withstands this pressure due to upregulation of stress response genes such as the *rpoS* regulon (6). In the intestine, *Salmonella* evades innate immune responses, such as bile salts and avian β-defensins, to interact with the epithelium (7, 8). *Salmonella* pathogenicity island 1 (SPI-1)- and SPI-2-encoded type-three secretion system 1 (T3SS-1) and T3SS-2 are used to invade tissue and to establish and maintain *Salmonella*-containing vacuoles in macrophages, respectively (9, 10).

Macrophages containing *Salmonella* organisms are the primary vessels for dissemination. To survive inside macrophages, *Salmonella* has to defend itself against many host killing factors: low Mg^{2+} and Ca^{2+}, acidic pH, and reactive oxygen species (7). To survive in this environment, *Salmonella* utilizes the components of T3SS-1 and T3SS-2 and the PhoP/PhoQ regulon (10–13). *S.* Enteritidis has been shown to colonize the ovary and the reproductive tract within laying hens, with a higher recovery from the ovary than from any other reproductive tissue (14, 15). Successful *S.* Enteritidis colonization involves inherent characteristics employed to subvert the reproductive innate immune system: phagocytes, antimicrobial peptides, including avian β-defensins, and immunoglobulins (16, 17). Persistent reproductive-tract colonization that leads to egg contamination is confined primarily to *S.* Enteritidis and is partially due to its ability to survive under these harsh conditions without causing overt clinical signs in the chicken host (18, 19). While much information has been gathered

Received 25 July 2014 **Returned for modification** 26 August 2014
Accepted 24 September 2014

Published ahead of print 29 September 2014

Editor: A. J. Bäumler

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doi:10.1128/IAI.02387-14

on the significance and mechanisms of T3SS-1 and T3SS-2, the mechanisms of persistence employed by *S.* Enteritidis remain to be fully understood (20).

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, accounting for 6.4% of the genome of *S.* Enteritidis and 9.6% of the genome of *S.* Typhimurium. One major difference is in the composition of their outer membranes or O-antigens (D1 for *S.* Enteritidis and B for *S.* Typhimurium), a key barrier to innate defenses and interaction with the host (21, 22). The studies aimed at understanding the mechanisms of persistence in the chicken host have often involved chicks. The data collected from these experiments do not transpose to infection of mature hens with *S.* Enteritidis, especially since the immunological landscape changes after the point of lay (23). In the present study, we identified *S.* Enteritidis genes overexpressed 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*. The current investigation revealed that AMPR genes used by *S.* Enteritidis to evade host innate immune defenses also play a role in reproductive-tract colonization and egg deposition.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Spontaneously nalidixic acid-resistant *S.* Enteritidis, designated strain ZM100, was generated by serial passages in Luria-Bertani (LB) broth with increasing concentrations of nalidixic acid. *S.* Enteritidis and *Escherichia coli* strains were cultured aerobically in tryptic soy broth (TSB), Super Optimal broth (SOB) or SOC (SOB plus 20 mM glucose [3.603 g]), or 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.

Cell cultures and culture conditions. Primary chicken oviduct epithelium cells (COEC) were prepared as described previously (10). Briefly, oviduct tissue (isthmus region) from 20- to 23-week-old Hy-line W36 chickens 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 at 200 U/ml and streptomycin at 200 mg/ml. After treatment with collagenase XI (Sigma) (1 mg/ml), the epithelial cells were released by treatment with 0.25% trypsin–EDTA (Invitrogen), collected via centrifugation at 100 × *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), 0.05 mM β-estradiol (Sigma), and 0.01 mg/ml insulin (Sigma). COEC were seeded into 48-well tissue culture plates at a density of 4×10^4 cells per well (for selective capture of transcribed sequences [SCOTS]) or into 96-well plates at a density of 2×10^4 cells per well (for invasion assays) and incubated at 37°C in 5% CO_2 for 48 h. COEC were stained with monoclonal antipancytokeratin antibody and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG and examined with an Olympus IX81 FA microscope. Cultures with more than 80% cytokeratin-positive (epithelial lineage) cells were used in subsequent infections. HD11 chicken macrophage cells (28) were maintained in RPMI 1640 tissue culture medium (Invitrogen) supplemented with 10% FBS and 2% CS at 37°C in 5% CO_2. Prior to infections, HD11 cells were seeded into 48-well tissue culture plates at a density of 4×10^5 cells per well (for SCOTS assays) or into 96-well plates at a density of 2×10^5 cells per well (for invasion assays) and incubated for 24 h.

Infection of cell cultures. Gentamicin protection assays were performed for invasion assays and SCOTS as described previously (29). To prepare the bacterial inoculum, 50 μl of an overnight culture of an *S.* Enteritidis strain (strain ZM100 only for SCOTS assays) 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, in experiments performed in triplicate, was washed three times in the appropriate media containing no antibiotics. For SCOTS assays, 200-μl bacterial suspensions containing approximately 8×10^5 CFU of logarithmic-phase ZM100 cells (for COEC) or 8×10^6 CFU stationary-phase ZM100 cells (for HD11) were added into each well to reach a multiplicity of infection (MOI) of 20:1 (bacteria/cells). For invasion and intracellular replication assays, 200-μl bacterial suspensions containing approximately 2×10^7 CFU of either logarithmic- or stationary-phase *S.* Enteritidis strains (for both COEC

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Description or relevant genotype[a]	Reference or source
Strains		
S. Enteritidis ZM100	Wild-type *S.* Enteritidis, Nal^r	24
S. Enteritidis ZM112	ZM100 Nal^r Cm^r *virK*::pEP185.2	This study
S. Enteritidis ZM114	ZM100 Nal^r Cm^r *ybjX*::pEP185.2	This study
S. Enteritidis ZM112C	ZM112 Nal^r Cm^r Amp^r pWSK*VirK*	This study
S. Enteritidis ZM114C	ZM114 Nal^r Cm^r Amp^r pWSK*YbjX*	This study
S. Enteritidis ZM122	ZM100 Nal^r Δ*virK* (Δ14–919/930)	This study
S. Enteritidis ZM123	ZM100 Nal^r Δ*ybjX* (Δ13–927/969)	This study
S. Enteritidis ZM124	ZM100 Nal^r Δ*virK* Δ*ybjX* (Δ14–919/930) (Δ13–927/969)	This study
S. Enteritidis ZM122C	ZM122 Nal^r Amp^r pWSK*VirK*	This study
S. Enteritidis ZM123C	ZM123 Nal^r Amp^r pWSK*YbjX*	This study
E. coli S17-1	*recA* Tn*7* λ*pir*	SZ collection
E. coli Top10F'	F′ *lacQ*^{Q} Tn*10* (Tet^r)	Invitrogen
Plasmids		
pCR2.1	TA cloning vector, Amp^r Kan^r *lacZ*α	Invitrogen
pEP185.2	Suicide vector, Cm^r	25
pRDH10	Cm^r, *sacB*	26
pWSK29	Low-copy-number expression vector, Amp^r	27
pZM-16S	pCR2.1 16S rRNA gene	This study
pZM-23S	pCR2.1 23S rRNA gene	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

[a] Amp, ampicillin; Cm, chloramphenicol; Kan, kanamycin; Nal, nalidixic acid; Tet, tetracycline.

and HD11) were added into the triplicate wells to reach an MOI of 20:1 for each well. To synchronize infections, all inoculated cultures were centrifuged at 800 × *g* for 10 min and then incubated at 37°C in 5% CO_2 for 1 h. Extracellular bacteria were removed by treatment with 100 μg/ml gentamicin in MEM (for COEC) or RPMI 1640 (for HD11) at 37°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 h and 15 h followed by lysis. These time points were designated 1 h postinfection (hpi) (T1), 4 hpi (T4), and 16 hpi (T16). 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, 10-fold serial dilutions of the invaded-cell lysates were plated onto LB agar supplemented with appropriate antibiotics and incubated overnight at 37°C for CFU enumeration. Invasiveness was calculated for each strain as the proportion of inoculum internalized at T1, and intracellular replication, or survival, was calculated as the proportion of *S.* Enteritidis cells recovered at T4 or T16 compared to the inoculum.

Preparation of bacterial gDNA and rRNA genes. Genomic DNA (gDNA) was isolated from an overnight ZM100 culture using a 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 (200-W) incandescent light for 30 min. The labeled gDNA was extracted with 2-butynal, washed with 70% ethanol, and resuspended in 10 mM Tris-HCl–0.5 mM EDTA (1× TE) buffer.

The 16S and 23S rRNA coding regions of *S.* Enteritidis were amplified by PCR using primer pair 16S-F1 and 16S-R1 and primer pair 23S-F1 and 23S-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 a Wizard Plus Minipreps DNA purification system (Promega) according to the manufacturer's instructions. The DNA concentration was determined based on the A_{260} spectrophotometer reading.

Isolation of RNA. Total RNA was isolated from strain ZM100-infected cell cultures and ZM100 grown in TSB using TRIzol reagents (Life Technologies). RNA samples were treated with RNase-free DNase I (Ambion), and the RNA concentration was determined by A_{260} and A_{280} spectrophotometer readings and agarose gel electrophoresis.

Synthesis of cDNA. RNA (5 μg) was converted to first-strand cDNA by random priming using a Superscript III system (Invitrogen) and primers ZM1-Nw and ZM2-Nw for intracellular and broth-grown bacteria, respectively. Double-stranded cDNA was generated using Klenow DNA polymerase (Promega) and amplified by PCR using primer ZM1-Nw (for intracellular bacteria) or primer ZM2-Nw (for broth-grown bacteria). 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, and 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 volumes of cDNA) with 3 M sodium acetate (NaOac) (0.1 volume of cDNA) and 1 μl glycogen (1 μg/ml) and resuspended in 10 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-3-propanesulfonic acid–1 mM EDTA (1× EPPS-EDTA). DNA concentrations were determined based on the A_{260} spectrophotometer reading.

SCOTS. To block rRNA coding regions, pZM-16S, pZM-23S (cloned rRNA gene), and biotinylated gDNA were mixed at a ratio of 5:5:1 (μg), fragmented by sonication, and resuspended in 1× EPPS-EDTA. The rRNA gene-blocked gDNA (8 μl) and the amplified cDNA (3 μg plus 8 μl 1× EPPS-EDTA) were denatured separately at 98°C for 3 min. Following addition of 2 μl 5 M NaCl to each reaction tube, the denatured gDNA and cDNA were self-annealed at 67°C for 30 min. The gDNA and cDNA were then 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, amplified cDNAs from the 10 parallel 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 under a given set of growth conditions. Following enrichments, competitive hybridizations were performed: rRNA gene-blocked and biotinylated gDNAs were prehybridized with cDNA derived from broth-grown bacteria at 67°C for 4 h and then hybridized with cDNA of intracellular bacteria for an 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 was cloned into pCR2.1. Following transformation of *E. coli* TOP10F′, the insertions of all clones were sequenced and compared to the *S. enterica* genomes using the BLASTN algorithm. Transcripts identified by two independent SCOTS procedures following two independent infections were considered intracellularly expressed.

RT-PCR. Reverse transcriptase PCR (RT-PCR) was conducted using MultiScribe reverse transcriptase (Invitrogen) and SYBR green PCR master mix (Applied Biosystems). The primer sequences of *Salmonella* genes were obtained from the Entrez Nucleotide database and are listed in Table 2. Reverse transcription of total RNA (2 μg) in a volume of 100 μl containing 5.5 mM $MgCl_2$, 500 μM deoxynucleoside triphosphate (dNTP), 2.5 μM random hexamers, and 1.25 U of MultiScribe reverse transcriptase was performed at 42°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, 58°C for 30 s, and 72°C for 30 s in the presence of 1× SYBR green PCR master mix (Applied Biosystems). The threshold cycle ($2^{-\Delta\Delta CT} \pm$ standard deviation [SD]) method was used to qualify transcriptional changes within host cells (30), and the expression of 16S rRNA was used to normalize the cDNA concentrations of different samples.

Construction of mutants. Mutants and complemented strains were constructed using the primers listed in Table 2. The initial insertion mutants, strains with *virK* (ZM112) or *ybjX* (ZM114) inserted, were constructed for inactivation of the target gene as previously described (31). Briefly, DNA fragments carrying the 5′ termini of *virK* and *ybjX* were amplified by PCR using primer pair virK-ORF1 and virK-R and primer pair ybjX-ORF1 and ybjX-rt-R, respectively. The PCR products were cloned into pCR2.1TOPO (Invitrogen) and subcloned into the SacI and XbaI sites of pEP185.2, a suicide vector coding for chloramphenicol resistance (25). 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. Exconjugants with a pEP185.2 insertion into the chromosome of strain ZM100 were selected by determination of resistance to chloramphenicol and nalidixic acid. These mutants were used in initial *in vitro* phenotypic characterizations. For complementation, the open reading frames (ORF) of *virK* and *ybjX* were amplified by PCR using primer pair VirK-ORF1 and VirK-ORF2 and primer pair YbjX-ORF1 and YbjX-ORF2. The PCR products were cloned directionally under the control of the *lac* promoter of pWSK29, a low-copy-number expression vector (27), which generated plasmids pWSK-*VirK* and pWSK*YbjX*. These plasmids were introduced into the corresponding mutant strains by electroporation and selection for resistance to ampicillin. The resultant strains were designated ZM112C and ZM114C, respectively.

To avoid possible polar effects, unmarked Δ*virK* (ZM122) and Δ*ybjX* (ZM123) deletion mutants and a Δ*virK* Δ*ybjX* (ZM124) double mutant were constructed using allelic exchange mutagenesis as previously de-

TABLE 2 Primers used in this study[a]

Primer	Sequence (5′–3′)	Amplicon size (bp)
For SCOTS		
ZM-1	GACACTCTCGAG ACATCACCGG	NA
ZM-2	TGCTCTAGACGTCGACATGGTT	
ZM1-Nw	GACACTCTCGAGACATCACTGG(N_9)	NA
ZM2-Nw	TGCTCTAGACGTCGACATGGTT(N_9)	
16S-F1	CGGACGGGTGAGTAATGTCT	1,372
16S-R1	ATCACAAAGTGGTAAGCGCC	
23S-F1	CGGGGGAACTGAAACATCTA	2,636
23S-R1	TCAACGTCGTCGTCTTCAAC	
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	

(Continued on following page)

TABLE 2 (Continued)

Primer	Sequence (5′–3′)	Amplicon size (bp)
virK-ORF1	GCGAGCTCATGACGATGCAGCAAAG	138
virK-R	AATAAGGCAACGTAATAC	
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. NA, not applicable.

scribed (31). Briefly, the upstream and downstream regions of the genes were amplified by PCR using primer pair virK-UF1 and virK-UR1, primer pair virK-DF1 and virK-DR1, primer pair ybjX-UF2 and ybjX-UR2, and primer pair ybjX-DF2 and ybjX-DR2, respectively. After ligation of the upstream and downstream products, the fusion was reamplified by PCR and cloned into pCR2.1TOPO. The fusion was then subcloned into vector pRDH10, a suicide vector that carries the *sacB* and chloramphenicol resistance genes and is λ*pir* dependent (26). The resulting plasmids, pZM122 and pZM123, were chemically transformed into *E. coli* S17-1λ*pir*, selected by resistance to chloramphenicol, and then transferred to ZM100 by conjugation. Exconjugants were selected by resistance to nalidixic acid and chloramphenicol for the insertion of the plasmid into the genome. Selection for a second recombination leading to unmarked deletion was done in 10% sucrose LB broth followed by growth on 5% sucrose LB plates at 30°C. The colonies sensitive to chloramphenicol were subjected to PCR to screen for the deletion of interest. Each unmarked deletion was complemented with its respective pWSK29 plasmid harboring the ORF of the gene as described above. The unmarked deletion for each gene was confirmed by DNA sequencing. These unmarked deletion mutants were used in further *in vitro* phenotypic characterizations as well as *in vivo* phenotypic characterizations. The strains and plasmids are listed in Table 1.

Bacterial cell morphology assay. Bacterial morphology for ZM100, Δ*virK*, Δ*ybjX*, Δ*virK* Δ*ybjX*, Δ*virK* pWSK*VirK* (ZM122C), and Δ*ybjX* pWSK*YbjX* (ZM123C) strains was determined by microscopic examination of logarithmic and stationary cultures at 4 h and 16 h of growth. Three fields were captured at 400× from three separate experiments by a Sony microscope camera and analyzed for the average Feret length using ImageJ morphometric analysis (32). After conversion to micrometers (12.156 pixels/μm), the data are presented as the average length per strain per time point.

Cell motility assay. To test the ability of the ZM100, Δ*virK*, Δ*ybjX*, Δ*virK* Δ*ybjX*, Δ*virK* pWSK*VirK*, and Δ*ybjX* pWSK*YbjX* strains to swim, bacteria were inoculated into 0.3% agar LB plates as previously described (33). Briefly, equal amounts of logarithmic-phase (4-h) cultures were spotted in the middle of 0.3% agar plates and incubated at 37°C for 3 h, at which point their motility diameter was measured. The data are represented as relative motility values (percentage of ZM100 motility).

EDTA and DOC sensitivity assays. The ZM100, Δ*virK*, Δ*ybjX*, Δ*virK* Δ*ybjX*, Δ*virK* pWSK*VirK*, and Δ*ybjX* pWSK*YbjX* strains were tested for their sensitivity to EDTA and deoxycholic acid (DOC) as previously described (34, 35). Briefly, approximately 1×10^8 CFU bacteria were added to warm 0.5% LB agar and the mixture was poured over 1.5% LB agar plates. Once the plates had dried, filter disks containing 0.5 M EDTA were placed in the center of the agar and incubated without inversion at 37°C for 16 h. The zones of inhibition were measured in millimeters, and the data are presented as relative sensitivity values (percent difference from ZM100). For the determinations of DOC sensitivity, LB agar plates containing 1% DOC and plain LB agar plates were inoculated with 10-fold serial dilutions of bacterial culture and incubated overnight at 37°C for enumeration. Percent inhibition of growth was calculated using the following formula: [(CFU on plain LB agar − CFU on 1% DOC LB agar)/(CFU on plain LB agar)] × 100.

Cell supernatant 2D SDS-PAGE assay. Cell supernatant proteins were obtained from the ZM100, Δ*virK*, Δ*ybjX*, and Δ*virK* Δ*ybjX* strains as previously described (36). Briefly, bacterial cultures were grown in triplicate overnight at 37°C and 250 rpm, subcultured at 1:50× into fresh LB broth, and incubated 4 h at 37°C and 250 rpm. Culture supernatants from approximately 12×10^9 CFU of each strain were recovered by centrifugation at 5,000 × *g* at 4°C for 15 min and filtration through a 0.45-μm-pore-size sterile filter. The supernatant was concentrated using centrifugal filters (molecular weight cutoff, 3,000), and the proteins in the supernatant were obtained by methanol-chloroform protein precipitation. A portion of the total supernatant proteins recovered was used to measure the concentration using a Bradford assay and was also visualized on a 12.5% SDS-PAGE gel stained with 0.1% silver stain to check for purity. One-fourth of the total supernatant proteins (representing 3×10^9 CFU per strain) were analyzed by two-dimensional (2D) SDS-PAGE. Nonlinear isoelectric focusing strips (pH 3 to 10, 7 cm in length) were rehydrated overnight with proteins dissolved in UT Chaps buffer consisting of 7 M urea, 2 M thiourea, and 4% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate} with DeStreak, pharmalytes, amylytes, and bromophenol blue (GE Healthcare), subjected to 770 V-h, and 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 differences between the Δ*virK*,

Δ*ybjX*, and Δ*virK* Δ*ybjX* deletion mutants and wild-type ZM100 in spot densities (32). Spots whose density was greater or lesser than 30% of the density of ZM100 were excised, reduced, and alkylated with iodoacetamide, digested with trypsin, and then processed on a ThermoFisher LTQ or OrbiTrap linear ion trap mass spectrometer using nano-liquid chromatography (nano-LC) peptide separations. Proteins were analyzed with Scaffold 4.1 for specific protein identities by using BLAST analysis and NCBI and UniProt databases (37).

Polymyxin B sensitivity assay. The *S.* Enteritidis ZM100, *virK*, *ybjX*, *virK* pWSK*VirK*, and *ybjX* pWSK*YbjX* strains were grown at 37°C in N-minimal media containing 10 mM MgCl as described previously (38). The pH of the medium was buffered with 100 mM Tris-HCl (pH 7.4). Approximately 5×10^5 CFU were inoculated into 1 ml LB containing 2.5 μg polymyxin B (Sigma) and incubated at 37°C for 1 h. Serial dilutions of bacterial suspensions were prepared in phosphate-buffered saline (PBS) and plated on LB agar plates for CFU enumeration. Polymyxin B sensitivity was expressed using the following equation: log reduction = [log input (CFU/ml) − log viability (CFU/ml)].

Avian beta-defensin sensitivity assay. The mature peptide of avian beta-defensin-6 (AvBD6) (SPIHACRYQRGVCIPGPCRWPYYRVGSCG SGLKSCCVRNRWA) with three disulfide bridges (Cys1-Cys5, Cys2-Cys4, and Cys3-Cys6) was synthesized by LifeTein LLC (Hillsborough, NJ). The sensitivity of *S.* Enteritidis strains to AvBD-6 was determined using a microbroth dilution method (39). In brief, overnight cultures were diluted in fresh Mueller-Hinton broth to achieve a 0.5 McFarland turbidity standard (approximately 5×10^7 CFU/ml). Equal volumes (50 μl of 5×10^5 CFU) of bacterial suspension and AvBD-6 (32 μg/ml) were mixed and incubated at 37°C for 1 h. For controls, PBS was used to replace AvBD-6. Following incubation, serial dilutions of bacterial suspensions were prepared in PBS and plated on LB agar plates for CFU enumeration. AvBD sensitivity was expressed as the percentage of growth inhibition relative to the growth of the AvBD-free control: [100 × (CFU of PBS-treated culture − CFU of AvBD-treated culture)/CFU of PBS-treated culture].

Ethics statement. Animal rearing, maintenance, and euthanasia were performed according the recommendations by the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and our Texas A&M University Institutional Animal Care and Use Committee SACC-approved Animal Use Protocol (permit number 2011-143).

Animal experiments. Female Hy-Line W36 chicks, obtained at 1 day of age from a local hatchery without vaccination for *S.* Enteritidis, were maintained at 30°C until they were 7 weeks old and were then moved to floor pens maintained at room temperature until they were 21 weeks old. They were given feed and water *at libitum*. Three hens were sacrificed prior to the experiments, and their spleen, oviduct, ovary, and cecum were tested by PCR to rule out *Salmonella*-positive status.

Prior to oral inoculations, the hens were housed in an animal biosafety level 2 (ABSL-2) housing facility with 2 hens per cage and grouped into six cage stacks so that seven groups of 12 hens were isolated from each other. While on a schedule of 16 h of light and 8 h of dark, the hens were given water and standard layer diet *ad libitum* for the duration of the infection. The overnight cultures of *S.* Enteritidis strains were diluted 1:50× with sterile LB broth and incubated for 16 h at 37°C and 250 rpm. The resultant cultures were harvested by centrifugation at 3,000 rpm at 4°C for 20 min and resuspended in sterile saline solution at a final concentration of 5×10^9 CFU/ml. Each group of 12 hens was orally inoculated with 1 ml of one bacterial strain per hen; one group of hens was inoculated with 1 ml of sterile saline solution to serve as a control. At the same time each day postinfection (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 euthanized and the spleen, oviduct (isthmus region), ovary, cecum, and ileum were collected and stored at −80°C.

For bacterial enumeration, approximately 1 g of feces was resuspended in 10 ml of buffered peptone water (BPW) and 10-fold serial dilutions were plated onto XLT4 agar followed by incubation at 37°C for 16 h. The BPW reaction mixture was then enriched overnight at 37°C and plated on XLT4 agar, and any growth seen was arbitrarily assigned a value of 5 CFU/g, the detection threshold for the procedure. The eggs were washed three times in 70% ethanol, placed in 50 ml of BPW, and homogenized for 5 min. A portion of the homogenized egg-BPW mixture was plated onto selective LB agar for enumeration, and the remainder was enriched for 2 days at 37°C, with plating of 24-h and 48-h enrichments performed on selective LB agar. Tissue samples were sterilely collected, weighed, homogenized for 30 s in 5 ml PBS, and plated on selective LB for CFU enumeration. The cecal and ileal contents were weighed, resuspended in 5 ml BPW, and then plated on XLT4 and selective LB for enumeration. The cecal and ileal tissues were washed three times in PBS, homogenized for 30 s in 5 ml PBS, and plated on selective LB for enumeration. Homogenates were enriched overnight at 37°C and plated on selective LB agar. Cultures that were negative in the initial plating but had growth after enrichment were arbitrarily assigned values of 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) × 5 ml]/4 and arbitrary cecum content CFU/homogenate = [(1 CFU/0.1 ml) × 5 ml]/4. When possible, three colonies from each culture with a positive result were tested by PCR to validate the accuracy of the visual counts. The data are expressed as log CFU/g for the feces and tissue and as log CFU/ovary for the ovary.

Statistics. Statistical analyses using one-way analysis of variance (ANOVA) were performed 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 ($P < 0.05$).

RESULTS

Detection of *S.* Enteritidis genes overexpressed in infected chicken cells by selective capture of transcribed sequences (SCOTS). To better understand the mechanism of *S.* Enteritidis colonization in chickens, SCOTS procedures were performed to identify the genes preferentially expressed in a 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. Of the 48 genes identified, 37 were overexpressed in COEC, 26 in HD11, and 15 in both types of cells (Table 3). The intracellular expression of selected genes was further confirmed by quantitative real-time PCR using 16S rRNA as a reference gene (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 included those involved in nitrate reduction, cell wall synthesis, and O-antigen conversion as well as the anaerobically induced *tdc* operon. *S.* Enteritidis genes overexpressed in both cell types comprised those encoding the SPI-2 T3SS apparatus, the SPI-5 *pipB* gene, and genes responsible for antimicrobial peptide resistance (AMPR) (*virK* and *ybjX*). Increased expression of the genes was detected in both cell types at 4 hpi, suggesting the significance of these genes in intracellular survival or replication inside COEC and HD11. In addition, several genes of unknown functions (such as *yifK* and *yjjZ*, hypothetically involved in transport, with the latter being overexpressed around 30-fold) were overexpressed in both types of cells (Fig. 1).

TABLE 3 Genes overexpressed by *S.* Enteritidis in chicken oviduct epithelial cells and macrophages[a]

Category	Gene	Description and possible function	COEC result		HD11 result	
			1 h	4 h	1 h	4 h
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		+		+
Antimicrobial peptide resistance	*pgtE*	Outer membrane protease E		+		+
	virK	Intracellular survival		+		+
	ybjX	Putative *virK* homologue		+		+
	fumA	Fumarate hydratase	+			
	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			+	
Membrane transport, metabolism, stress response	*narU-narY*	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-yraP	Putative phosphoheptose isomerase		+		
	trpS	Tryptophanyl-tRNA synthetase		+		+
	tdcB	L-Threonine/L-serine permease, anaerobically inducible				+
	tdcC-tdcD	Catabolic threonine dehydratase, anaerobic metabolism				+
Restriction modification	*spa1514*	Putative DNA/RNA-nonspecific endonuclease	+			
	hsdM	DNA methylase, protecting DNA against endonuclease	+			
	hsdS	DNA methylase, protecting DNA against endonuclease	+			
	hsdR	DNA methylase, protecting DNA against endonuclease	+			
Cell wall and surface structure	*sefB*	Fimbrial periplasmic chaperone, pilus 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		+		

[a] COEC, chicken oviduct epithelial cells; HD11, macrophages.

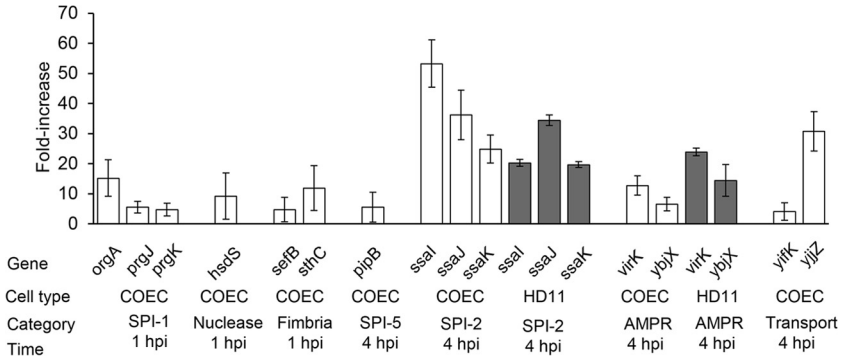

FIG 1 Quantitative analysis of genes overexpressed by *S.* Enteritidis upon infection in COEC and HD11 cells. Relevant genes found to be overexpressed in COEC or HD11 cells from the SCOTS experiment were quantified for their intracellular expression in these cell types using reverse transcriptase real-time PCR; the fold increase is shown ($2^{-\Delta\Delta CT} \pm$ SD, where SD is the standard deviation). Data shown are from three independent experiments performed with duplicate runs per assay. For each gene, the cell type, category, and time postinfection at which the expression occurred are shown under the graph.

Sensitivity of the *S.* Enteritidis Δ*ybjX* mutant to EDTA and bile acids. To determine the sensitivity of wild-type (ZM100), Δ*virK* (ZM122), Δ*ybjX* (ZM123), and Δ*virK* Δ*ybjX* (ZM124) *S.* Enteritidis to EDTA, each strain was seeded into LB agar and tested for its ability to grow in the presence of a filter disc containing 0.5 M EDTA. The zones of inhibition were measured and are shown as representative of relative sensitivity levels (Fig. 2A). The Δ*ybjX* mutant, but not the Δ*virK* and Δ*virK* Δ*ybjX* mutants, was significantly more sensitive to 0.5 M EDTA (9.1% more sensitive than the wild type; $P < 0.05$). Growth in 0.5 M EDTA was restored to the wild-type phenotype when pWSK*YbjX* was introduced into the corresponding mutant strain. To test the sensitivity of strains to bile acid, bacteria were grown in LB agar containing 1% deoxycholic acid (1% DOC). As shown in Fig. 2B, the Δ*virK* Δ*ybjX* mutant was significantly more susceptible (99.6% killed; $P <$ 0.05) than any other strain to 1% DOC. These data indicate that AMPR genes play distinct roles in the susceptibility of *S.* Enteritidis to EDTA and 1% DOC, with *ybjX* having a greater role in this defense.

Altered cell morphology and motility of *S.* Enteritidis Δ*ybjX* and Δ*virK* Δ*ybjX* mutants. To characterize the functional contributions of AMPR genes to *S.* Enteritidis pathogenicity, we examined the differences between the wild-type and mutant *S.* Enteritidis strains in morphology and motility. During the logarithmic phase of growth and the stationary phase of growth (4 h and 16 h, respectively), the Δ*ybjX* and Δ*virK* Δ*ybjX* mutants, but not the Δ*virK* mutant, formed long filaments with average lengths (μm) and chain-forming tendencies exceeding those of the wild-type strain (Fig. 3A; $P < 0.05$). At logarithmic phase, the Δ*virK* Δ*ybjX* mutant exhibited a greater length (4.3 μm) than any of the other strains. Wild-type morphology was restored to the Δ*ybjX* mutant when a cloned *ybjX* gene (pWSK*YbjX*) was introduced into the strain (Fig. 3A). Regardless of the variations in cell length, all three mutants showed significantly decreased motility (81.7% compared to 85.4% ZM100 motility; $P < 0.05$) (Fig. 3B). The motility defects of the Δ*virK* and Δ*ybjX* mutants were partially restored when the mutants were complemented with pWSK*VirK* and pWSK*YbjX*, respectively (Fig. 3B).

Altered profiles of secreted proteins of *S.* Enteritidis AMPR mutants. The increased susceptibility of AMPR mutants to EDTA and deoxycholic acid along with the increased bacterial cell length suggested an altered outer membrane. To evaluate the integrity of

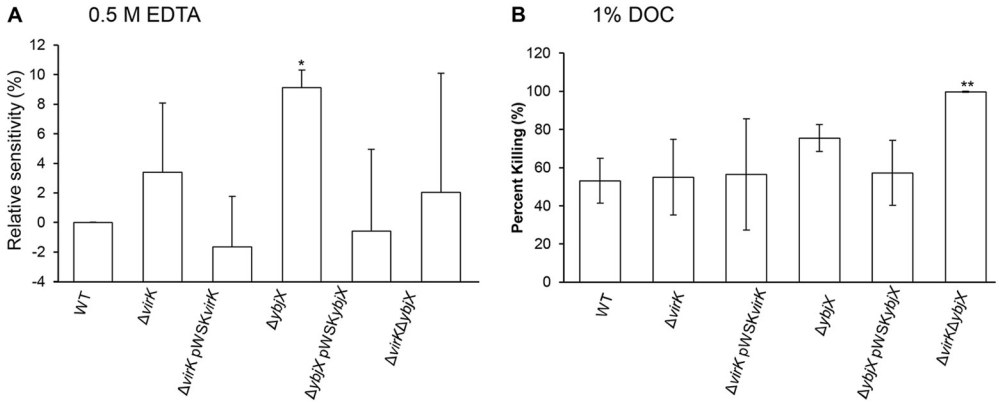

FIG 2 Sensitivity of AMPR mutants to EDTA and bile acid. Wild-type *S.* Enteritidis (WT; ZM100) and *S.* Enteritidis Δ*virK* (ZM122), Δ*ybjX* (ZM123), Δ*virK* Δ*ybjX* (ZM124), Δ*virK* pWSK*VirK* (ZM122C), and Δ*ybjX* pWSK*YbjX* (ZM123C) were tested for their sensitivity to EDTA and bile acid. (A) Sensitivity to 0.5 M EDTA was measured as zones of inhibition, and data are presented as percent WT inhibition. The Δ*ybjX* mutant exhibited significantly increased sensitivity to 0.5 M EDTA. (B) Sensitivity to bile acid was measured by comparing bacterial growth in LB to that in LB containing 1% DOC, and data are presented as percent killing by DOC. The Δ*virK* Δ*ybjX* mutant was significantly more sensitive than the WT strain to 1% DOC. Data shown are from three independent experiments. A single asterisk denotes statistical significance at $P < 0.05$, and double asterisks denote statistical significance at $P < 0.01$.

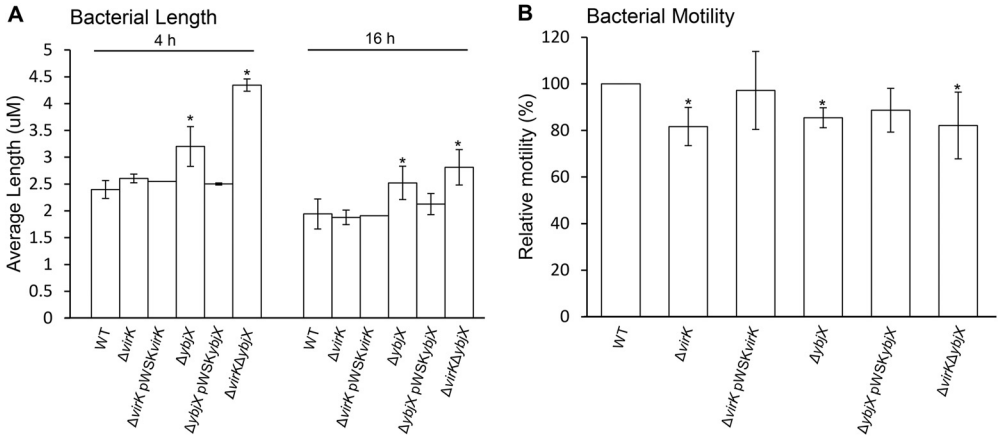

FIG 3 Average length and motility of AMPR mutants. (A) Wild-type *S.* Enteritidis (WT; ZM100) and *S.* Enteritidis Δ*virK* (ZM122), Δ*ybjX* (ZM123), Δ*virK* Δ*ybjX* (ZM124), ΔvirK pWSK*virK* (ZM122C), and Δ*ybjX* pWSK*YbjX* (ZM123C) were grown to log phase (4 h) and stationary phase (16 h), photographed at ×400 magnification, and analyzed for average Feret length using ImageJ morphometric analysis. The Δ*ybjX* and Δ*virK* Δ*ybj*X strains exhibited increased average lengths in both phases of growth. (B) Log-phase *S.* Enteritidis strains were inoculated onto 0.3% agar, and the swimming diameter (mm) of each strain was determined. Relative motility levels were determined following 3 h of incubation and are presented as percentages of WT motility. All three AMPR mutants showed decreased motility compared to WT strain ZM100. Data shown are from three independent experiments with duplicates in each assay. A single asterisk denotes statistical significance ($P < 0.05$).

the outer membrane of the AMPR mutants, we chose to assess their ability to appropriately secrete proteins. We extracted total proteins from the supernatants of the following log-phase *S.* Enteritidis strains: the wild type (ZM100), the Δ*virK* mutant (ZM122), the Δ*ybjX* mutant (ZM123), and the Δ*virK* Δ*ybjX* mutant (ZM124). We separated the supernatant proteins using 2D SDS-PAGE and analyzed the differences in protein spot densities. The 2D SDS-PAGE analysis revealed differences in the protein quantities between the wild-type strain and the mutant strains, especially the Δ*virK* Δ*ybjX* mutant (Fig. 4). We initially chose spots that exhibited at least a 30% difference between the wild-type and mutant strain results in densitometric analysis. Selected spots were excised and sequenced by LC-tandem mass spectrometry (LC-MS/MS) to determine their identities. The flagellum-associated proteins (FliC, FliK, FlgD, and FlgE) and *Salmonella* T3SS-1 invasion protein (SipD) were most abundant in the wild-type supernatant, decreased in abundance the supernatants of the Δ*virK* and Δ*ybjX* mutants, and most strikingly decreased in abundance in the supernatant of the Δ*virK* Δ*ybjX* mutant. The FliC protein, phase 1 flagellin, was not detectable in any of the 2D SDS-PAGE gels of the Δ*ybjX* and Δ*virK* Δ*ybjX* mutants. The supernatants of the Δ*virK* Δ*ybjX* and Δ*ybjX* mutants contained more bacterial cell wall proteins or plasma proteins, including EF Tu, a cell membrane-associated elongation factor Tu, OsmY, a

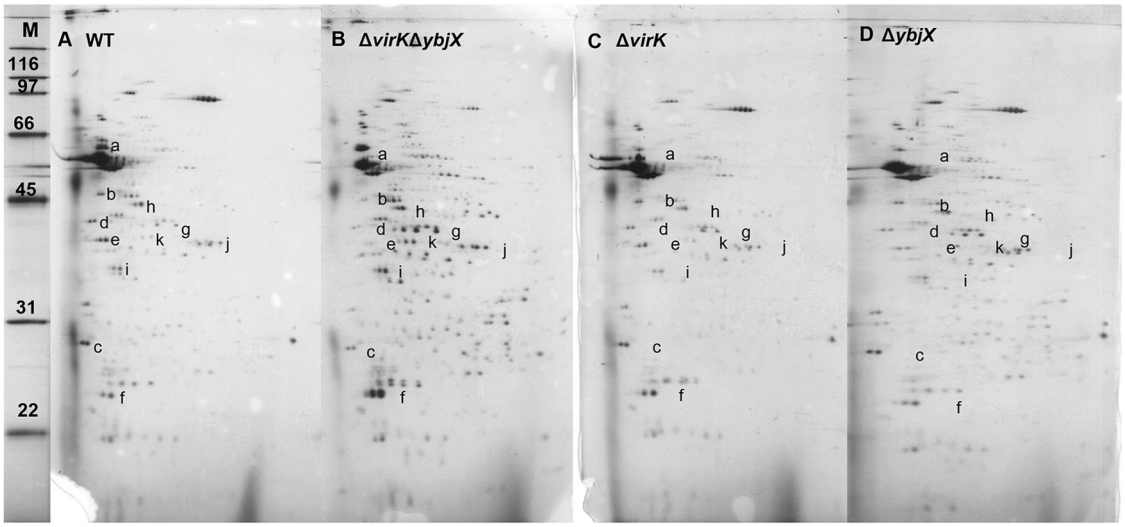

FIG 4 Differences in *in vitro* protein secretion. The proteins secreted into the culture supernatant from the log-phase wild-type strain and the AMPR mutants were extracted, separated using 2D SDS-PAGE, and analyzed densitometrically. The Δ*virK* Δ*ybjX* mutant displayed the greatest differences in spot densities (indicated by lowercase letters) compared to the wild-type strain. Representative 2D SDS-PAGE gels are shown as follows: lane M, protein ladder (kDa); lane A, wild-type *S.* Enteritidis (ZM100); lane B, *S.* Enteritidis Δ*virK* Δ*ybjX* (ZM124); lane C, *S.* Enteritidis Δ*virK* (ZM122); lane D, *S.* Enteritidis Δ*ybjX* (ZM123). Experiments were repeated three times with triplicate cultures in each run.

TABLE 4 *S.* Enteritidis proteins that differ in their levels of *in vitro*-secreted abundance between the wild-type strain and AMPR mutants

Spot ID[a] (see Fig. 5)	Molecular mass (kDa)	Gene symbol	Description	Cellular location	Protein coverage (%)	GenBank ID	Order of secreted protein abundance[b]
a	52	*fliC*	Phase-1 flagellin	Secreted	28.0	AAA27085.1	WT > Δv[c]
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	Cell 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] ID, identifier.
[b] The order of protein abundance was determined densitometrically from three 2D SDS-PAGE gels for the following *S.* Enteritidis strains: the wild type (WT), Δ*virK* mutant (Δv), Δ*ybjX* mutant (Δy), and Δ*virK* Δ*ybjX* mutant (ΔvΔy).
[c] FliC protein secretion was not detectable in the Δ*ybjX* (Δy) and Δ*virK* Δ*ybjX* (ΔvΔy) mutants with our methods.

periplasmic osmotically inducible protein, MalE, a periplasmic maltose transporter protein, GlpQ, a periplasmic glycerophosphoryl diester phosphodiesterase, EF Ts, a cytosolic elongation factor Ts, and GapA, a cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fig. 4 and Table 4).

Increased susceptibility of *S.* Enteritidis AMPR mutants to antimicrobial peptides. To assess the contributions of AMPR genes to antimicrobial peptide resistance, a series of bacterial growth inhibition or killing assays were performed. Treatment of *S.* Enteritidis strains with polymyxin B, a potent antimicrobial peptide, resulted in a 4-log reduction of the wild-type strain level and about 4.4-log reductions of the levels of the Δ*virK* and Δ*ybjX* mutants (Fig. 5A). Subsequently, *S.* Enteritidis strains were treated with AvBD-6, which resulted in growth inhibitions of 24.1% and 30% for *virK* and *ybjX* mutant strains, respectively, compared to 8.8% for the wild-type *S.* Enteritidis strain (Fig. 5B). Introduction of pWSK*virK* and pWSK*YbjX* into the corresponding mutant strains complemented the increased sensitivity to polymyxin B and AvBD-6. These results indicate that both *virK* and *ybjX* contribute to the resistance of *S.* Enteritidis to antimicrobial peptides, an important arm of the host innate immune system.

Altered interactions of *S.* Enteritidis AMPR mutants 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).

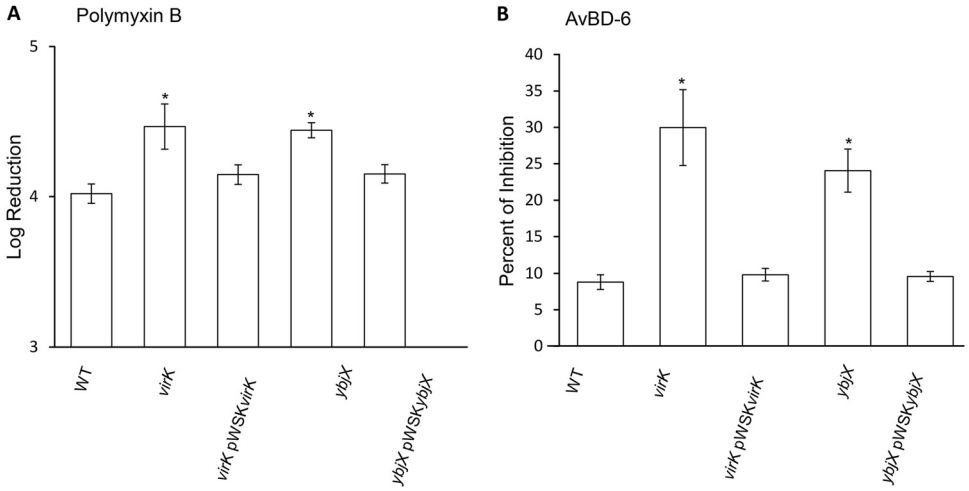

FIG 5 Sensitivity of AMPR mutants to antimicrobial peptides. Wild-type *S.* Enteritidis (ZM100) and *S.* Enteritidis *virK* (ZM112), *ybjX* (ZM114), *virK* pWSK*virK* (ZM112C), and *ybjX* pWSK*YbjX* (ZM114C) were tested for their sensitivity to antimicrobial peptides. (A) *S.* Enteritidis strains were exposed to a general, potent antimicrobial peptide, polymyxin B, for 1 h and then plated for CFU enumerations. Results are presented as the log reduction in CFU. *virK* and *ybjX* mutants showed significantly increased susceptibility. (B) *S.* Enteritidis strains were exposed to a host-specific antimicrobial peptide, AvBD-6, using a microbroth dilution method. Sensitivity to AvBD-6 is expressed as percent growth inhibition. Both the *virK* mutant and the *ybjX* mutant had increased susceptibility to AvBD-6. Data shown are from three independent experiments, and the average values are shown in parentheses. A single asterisk denotes significant differences ($P < 0.05$).

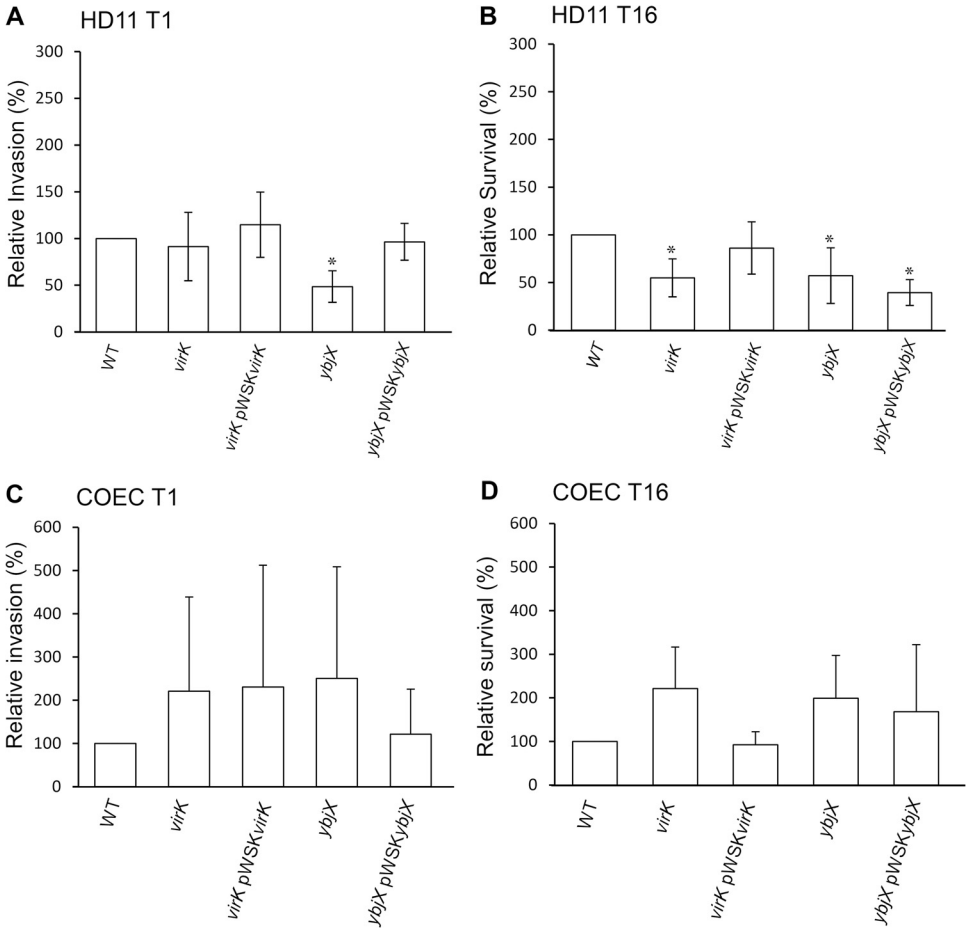

FIG 6 Relative invasion (or entry) and survival of AMPR mutants in chicken macrophage and reproductive epithelial cells. HD11 chicken macrophages and COEC were infected with stationary-phase wild-type *S.* Enteritidis (WT; ZM100) and *S.* Enteritidis *virK* (ZM112), *ybjX* (ZM114), *virK* pWSK*VirK* (ZM112C), and *ybjX* pWSK*YbjX* (ZM114C), and a gentamicin protection assay was performed. *S.* Enteritidis bacteria recovered after 1 hpi were analyzed to determine their ability to enter macrophages (A) or COEC (C); the results are shown as percentages of the WT CFU index counts. *S.* Enteritidis bacteria recovered after 16 hpi were analyzed to determine their ability to survive inside macrophages (B) or COEC (D); the results are shown as averages of the percentages of the WT recovery levels. Each assay was run in triplicate and repeated three times. A single asterisk denotes statistical significance ($P < 0.05$).

Our results showed that *virK* was not involved in the entry of *S.* Enteritidis into HD11 macrophages (Fig. 6A). In contrast, disruption of *ybjX* resulted in significantly decreased entry into HD11 cells (45% of wild type; $P < 0.05$). The defect demonstrated by the *ybjX* mutant was fully restored by introducing pWSK*YbjX* into the *ybjX* mutant (Fig. 6A). At 16 hpi, lower numbers of intracellular bacteria were recovered from HD11 cells infected with the *virK* and *ybjX* mutants than from those infected with the wild type (Fig. 6B; $P < 0.05$). The survival defect of the *virK* mutant, but not that of the *ybjX* mutant, was partially complemented by the cloned gene. The *ybjX* and *virK* mutants did not show any significant defect in entry of and intracellular survival in COEC (Fig. 6C and D).

Reduced ability of *S.* Enteritidis AMPR mutants to survive in the intestinal lumen and to shed in feces. To investigate the contribution of *virK* and *ybjX* genes to intestinal colonization and environmental spread during *S.* Enteritidis infection in chickens, we collected fecal samples at the same time every day following oral inoculation of 21-week-old laying hens with 5×10^9 CFU of wild-type or mutant strains. At 5 and 10 dpi, we cultured ileal and cecal tissue samples and the contents of the euthanized hens. We did not observe any significant differences in tissue bacterial loads among experimental groups (Fig. 7). In contrast, the bacterial loads of the Δ*virK* Δ*ybjX* mutant in cecal content were significantly lower than those of the wild-type strain (Fig. 8B; $P < 0.05$). Infection with the Δ*virK* Δ*ybjX* mutant also resulted in a significant decrease in fecal *S.* Enteritidis loads (Fig. 9A; $P < 0.05$) from 1 dpi to 5 dpi and significantly reduced number of shedders throughout the course of infection (Table 5). The compromised ability of the Δ*virK* Δ*ybjX* mutant to survive in cecal content from 1 dpi to 5 dpi coincided with reduced fecal bacterial loads and reduced numbers of shedders. Decreased fecal *Salmonella* load was also detected following infection with the Δ*ybjX* pWSK*YbjX* mutant (Fig. 9A), suggesting a possibility of compromised survival of this strain in the fecal environment.

S. Enteritidis AMPR genes involved in reproductive-tract colonization and egg contamination. To investigate the roles played by AMPR genes during *S.* Enteritidis colonization of the reproductive tract of laying hens and, ultimately, contamination of eggs, we collected eggs at the same time every day and ovaries and oviduct (isthmus) tissues at 5 dpi and 10 dpi from orally inoculated hens. The egg and tissue samples were tested for the presence and bacterial load of *S.* Enteritidis.

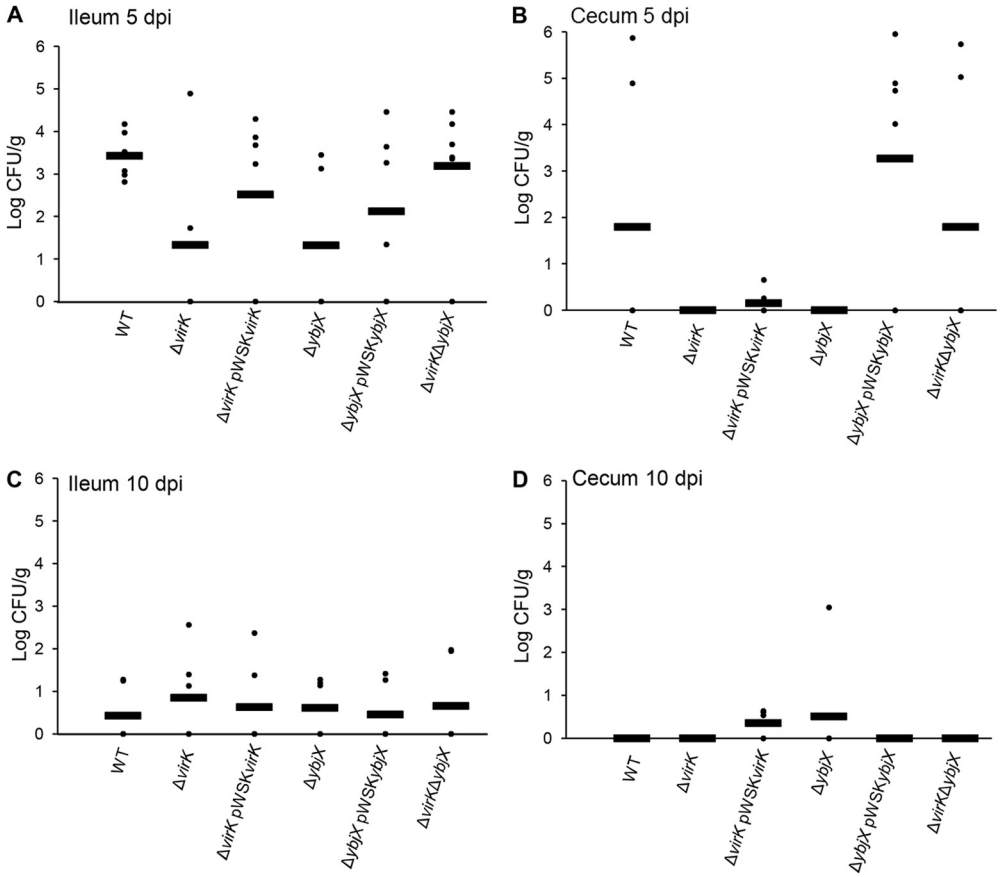

FIG 7 *S.* Enteritidis bacterial loads in intestinal tissue. Wild-type (ZM100) and Δ*virK* (ZM122), Δ*ybjX* (ZM123), Δ*virK* Δ*ybjX* (ZM124), Δ*virK* pWSK*VirK* (ZM122C), and Δ*ybjX* pWSK*YbjX* (ZM123C) bacteria were used to orally infect 21-week-old laying hens (12 hens per strain) at a dose of 5×10^9 CFU per hen. *S.* Enteritidis intestinal loads were determined at 5 and 10 dpi. No statistical significance of the results of comparisons of WT and mutant strains was observed. (A) Ileum at 5 dpi ($P_{virK} = 0.0546$ and $P_{ybjX} = 0.0556$). (B) Cecum at 5 dpi ($P_{virK} = 0.1186$ and $P_{ybjX} = 0.1186$). (C) Ileum at 10 dpi ($P > 1$). (D) Cecum at 10 dpi ($P_{ybjX} = 0.1022$). Dots represent individual bird bacterial loads, and bars represent the averages.

At 5 dpi, significantly reduced numbers of *S.* Enteritidis were recovered from the ovaries of the hens infected with the Δ*virK* and Δ*virK* Δ*ybjX* mutants compared to those infected with the wild-type strain (Fig. 10B; $P < 0.05$). In contrast, the Δ*ybjX* mutant did not show a defect in ovary colonization at 5 dpi. At 10 dpi, no significant difference in ovary bacterial load was observed among treatment groups. No significant difference in oviduct bacterial load was observed among treatment groups at 5 dpi and 10 dpi.

As shown by analysis of *S.* Enteritidis-positive ovaries at 5 dpi (Table 6), the wild-type strain infected the greatest proportion of ovaries (83.3%) and the Δ*virK* and Δ*virK* Δ*ybjX* mutants infected the lowest proportion of ovaries (16.7%). At 10 dpi, the Δ*ybjX* mutant infected the greatest proportion of ovaries (66.7%) whereas the Δ*virK* and Δ*virK* Δ*ybjX* mutants infected as many as the wild type (33.3%). Comparing these data to the percentages of contaminated eggs, we noticed that the Δ*ybjX* mutant was the only strain isolated from egg contents from 6 dpi to 9 dpi. The complemented Δ*ybjX* strain (ZM123C) was less able to contaminate eggs and to colonize ovaries (Table 6). The data suggest that AMPR genes play distinct roles in reproductive-tract colonization, with *virK* contributing to reproductive-tract colonization and egg deposition during the early stages of infection and *ybjX* playing a counteractive role in reproductive persistence and egg deposition.

DISCUSSION

The main vehicle of *S.* Enteritidis infection in humans is thought to be contaminated 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 persistent colonization of the reproductive tract of laying hens without inducing overt clinical symptoms (17). While many studies have confirmed that *S.* Enteritidis exploits inherent differences to purposefully colonize the reproductive tract and contaminate eggs better than other serovars, the exact mechanisms for this action have yet to be discovered (15, 17, 18, 40).

Many experiments performed with *S.* Enteritidis focus on the mechanisms of the T3SS-1 and T3SS-2 during invasion and intracellular replication within various chicken tissues (10, 41–43). Recent studies have focused on survival strategies of *S.* Enteritidis under the stressful conditions encountered by pathogens of the chicken such as those present in egg white. Those 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 (44, 45). In the present study, we used a

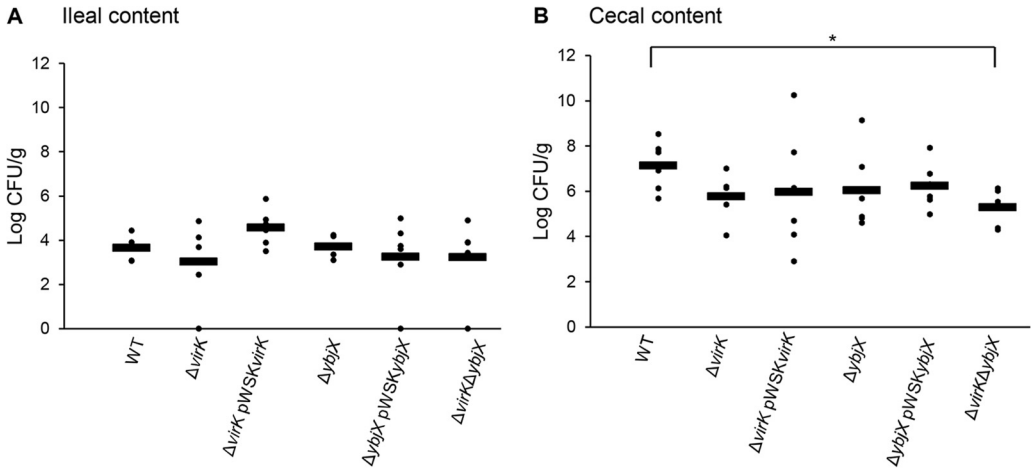

FIG 8 *S.* Enteritidis bacterial load in intestinal content. Wild-type (ZM100), Δ*virK* (ZM122), Δ*ybjX* (ZM123), Δ*virK* Δ*ybjX* (ZM124), Δ*virK* pWSK*VirK* (ZM122C), and Δ*ybjX* pWSK*YbjX* (ZM123C) bacteria were used to orally infect 21-week-old laying hens (12 hens per strain) with 5×10^9 CFU per hen. The number of *S.* Enteritidis bacteria in intestinal content was determined at 5 dpi. A significant difference between the WT strain and the Δ*virK* Δ*ybjX* double-mutation mutant in bacterial loads in the cecal content (B) was detected (* denotes $P < 0.05$). Dots represent individual bird loads, and bars represent the averages.

selective capture of transcribed genes (SCOTS) assay to identify the genes overexpressed by *S.* Enteritidis upon entry and survival in chicken macrophages (HD11) and chicken oviduct epithelial cells (COEC) and characterized those genes that were identified as antimicrobial peptide resistance genes (AMPR genes).

Salmonella enterica utilizes macrophages as a transport vessel to invade systemic sites within the host (46). Once *S.* Enteritidis invades the reproductive tract, successful egg contamination by *S.* Enteritidis most likely happens during egg development, with *S.* Enteritidis colonization predominantly found in the isthmus of the oviduct (15, 47). 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 overexpressed in HD11 and COEC consisted of a cohort of stress response, transport, cell wall and DNA modification, fimbrial, AMPR, and virulence genes. The identification of SPI-1 genes at 1 hpi and an SPI-2 gene at 4 hpi validated the authenticity of the results of the SCOTS experiment, as these genes are known to be involved in invasion and intracellular replication, respectively (11, 20, 48). The overexpression of SPI-2, SPI-5, and AMPR genes was 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 *pipB* gene have been studied for their effects during *S.* Enteritidis infection in hens, but there is a lack of knowl-

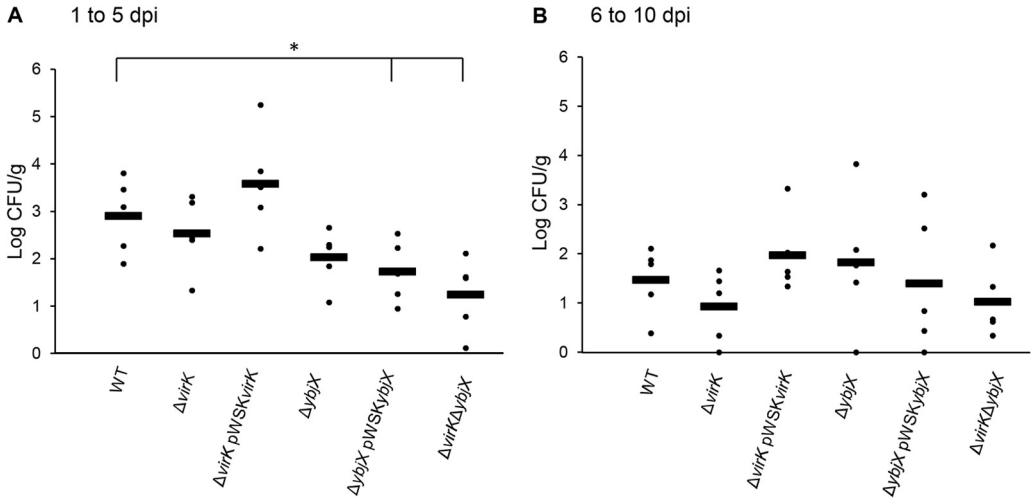

FIG 9 Fecal bacterial load of AMPR mutants during the early and late stages of infection. Wild-type *S.* Enteritidis (ZM100) and *S.* Enteritidis Δ*virK* (ZM122), Δ*ybjX* (ZM123), Δ*virK* Δ*ybjX* (ZM124), Δ*virK* pWSK*VirK* (ZM122C), and Δ*ybjX* pWSK*YbjX* (ZM123C) were used to orally infect 21-week-old laying hens (12 hens per strain) at a dose of 5×10^9 CFU per hen. A sample of feces was collected daily from each bird. Tenfold dilutions of samples in peptone-buffered water were plated to determine fecal bacterial loads. (A) Fecal bacterial loads from 1 to 5 dpi. (B) Fecal loads from 6 to 10 dpi. Significant differences in fecal loads collected from 1 to 5 dpi were detected (a single asterisk denotes $P < 0.05$). Each dot represents the average bacterial load of each strain per dpi, and each bar represents the overall average from 5 days.

TABLE 5 Hens shedding *S.* Enteritidis strains in their feces each day postinfection (dpi)[a]

Strain	No. of hens shedding *S.* Enteritidis strains on indicated dpi/total no. of hens (% positive)										% avg
	1	2	3	4	5	6	7	8	9	10	
S. Enteritidis WT	4/6 (66.7)	4/6 (66.7)	4/6 (66.7)	4/6 (66.7)	4/6 (66.7)	4/6 (66.7)	3/6 (50.0)	1/6 (16.7)	4/6 (66.7)	3/6 (50.0)	58.3
S. Enteritidis Δ*virK*	1/6 (16.7)	4/6 (66.7)	5/6 (83.3)	5/6 (83.3)	4/6 (66.7)	3/6 (50.0)	2/6 (33.3)	0/6 (0.0)	3/6 (50.0)	1/6 (16.7)	46.7
S. Enteritidis Δ*virK* pWSK*VirK*	5/6 (83.3)	5/6 (83.3)	3/6 (50.0)	6/6 (100.0)	3/6 (50.0)	5/6 (83.3)	3/6 (50.0)	2/6 (33.3)	2/6 (33.3)	3/6 (50.0)	61.7
S. Enteritidis Δ*ybjX*	3/6 (50.0)	3/6 (50.0)	4/6 (66.7)	2/6 (33.3)	4/6 (66.7)	3/6 (50.0)	5/6 (83.3)	2/6 (33.3)	0/6 (0.0)	3/6 (50.0)	48.3
S. Enteritidis Δ*ybjX* pWSK*YbjX*	3/6 (50.0)	3/6 (50.0)	3/6 (50.0)	2/6 (33.3)	3/6 (50.0)	4/6 (66.7)	4/6 (66.7)	2/6 (33.3)	1/6 (16.7)	1/6 (16.7)	41.7
S. Enteritidis Δ*virK* Δ*ybjX*	2/6 (33.3)	2/6 (33.3)	3/6 (50.0)	1/6 (16.7)	1/6 (16.7)	1/6 (16.7)	3/6 (50.0)	2/6 (33.3)	4/6 (66.7)	1/6 (16.7)	33.3*

[a] Fecal samples were collected daily during the infection challenge of 21-week-old laying hens with the *S.* Enteritidis strains. One gram of feces was cultured to determine fecal shedding. The fecal samples from 1 dpi to 5 dpi represented two hens, and the samples from 6 dpi to 10 dpi represented one hen. A single asterisk denotes a significant difference in the average number of shedding hens inoculated with the double mutant versus the WT ($P < 0.05$).

edge of the role AMPR genes *virK* and *ybjX* play during *S.* Enteritidis infection in chickens (10, 24, 42, 43).

The AMPR *virK* and *ybjX* genes 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 under low-Mg^{2+} conditions (13, 49–52). In *Shigella flexneri*, *virK* is hypothesized to modulate the outer membrane to alter the interaction between IcsP (an actin-modulating protein) and lipid A (52). In *S.* Typhimurium, a mutation in *ybjX* (initially termed *somA*) was found as a suppressor mutation corresponding to a mutation in the MsbB lipid A assembly protein, functionally linking *ybjX* to outer membrane modification (53). The outer membrane serves several bacterial functions, including stabilization for various functions, such as movement and secretion, and defense against host killing tactics. Mutations that affect outer

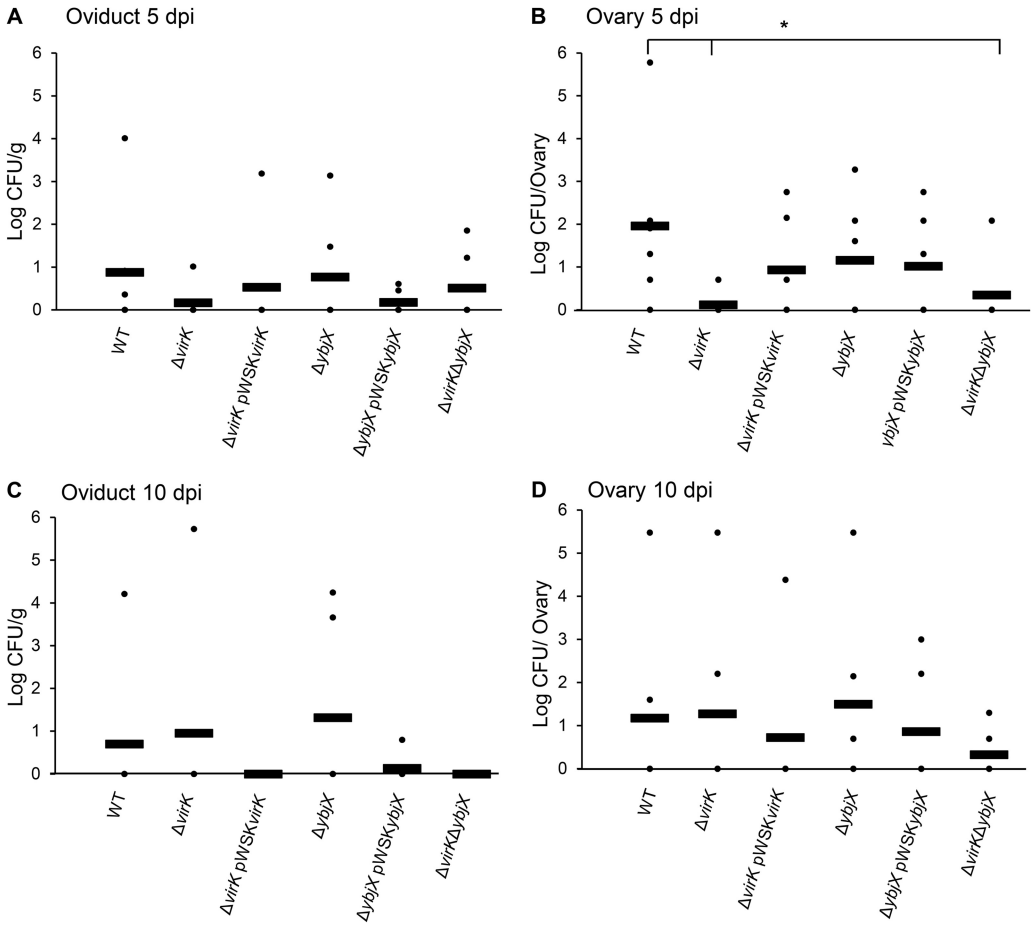

FIG 10 The bacterial loads of AMPR mutants in reproductive tissues. Wild-type *S.* Enteritidis (ZM100) and *S.* Enteritidis Δ*virK* (ZM122), Δ*ybjX* (ZM123), Δ*virK* Δ*ybjX* (ZM124), Δ*virK* pWSK*VirK* (ZM122C), and Δ*ybjX* pWSK*YbjX* (ZM123C) were used to orally infect 21-week-old laying hens (12 hens per strain) at a dose of 5×10^9 CFU per hen. Oviducts (isthmus) and ovaries were collected at 5 and 10 dpi, homogenized, and plated to determine *S.* Enteritidis loads. (A) Oviduct (isthmus) at 5 dpi. (B) Ovary at 5 dpi. (C) Oviduct (isthmus) at 10 dpi. (D) Ovary at 10 dpi. Each dot represents the bacterial load in an individual bird, each bar represents the average bacterial load in an individual group, and a single asterisk denotes statistical significance ($P < 0.05$).

membrane stability make the bacteria sensitive to detergents, to which *Salmonella* is naturally resistant (8, 24). 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 Mg^{2+} and Ca^{2+} cations that link the outer membrane lipopolysaccharide (LPS) molecules. To overcome the reduction in connective cation levels caused by EDTA, *Salmonella enterica* must actively employ membrane-stabilizing mechanisms, such as addition of myristic acid to the lipid A portion of LPS, to maintain a strong barrier (53). The inability to add more stabilizing fatty acids to anchor LPS molecules in the outer membrane results in increased susceptibility to EDTA (53). 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 (54). Deoxycholate is a bile acid, an anionic detergent, which can insert into the bacterial cell membrane, resulting in bacterial death. *Salmonella* organisms possess an outer membrane that is naturally resistant to the insertion of this detergent (8). Therefore, any alterations in the outer membrane, possibly caused by Δ*ybjX* and Δ*virK* Δ*ybjX*, would lead to increased susceptibility to deoxycholate.

In *S.* Typhimurium, a mutation in *msbB* caused formation of elongated cells and a mutation in *tatB* or *tatC* (encoding twin arginine transport proteins required for transporting outer membrane components) caused long aggregate filaments to form (34, 53). 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 in the previous study (53). In the current investigation, we observed an increase in cell length and filamentous formations for the Δ*ybjX* and Δ*virK* Δ*ybjX* mutants (Fig. 3). Therefore, the similar morphologies 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 require a stable outer membrane (33, 55, 56). The present study showed 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-deletion (Δ*virK* Δ*ybjX*) mutant displayed motility defects similar to those of the individual (Δ*virK* and Δ*ybjX*) mutants (Fig. 3). In *S.* Typhimurium, a mutation in *msbB* (*waaN*) resulted in an inability to secrete *Salmonella* effector proteins (36, 53). The current study showed that mutations in AMPR genes affect the ability to secrete proteins required for flagellar function (Fig. 5 and Table 4). FliC, FliK, FlgD, and FlgE proteins are secreted through a secretion system similar to the type three and type five secretion systems (56, 57). The inability to secrete these flagellar proteins may explain the decreased motility seen in the three AMPR mutants (58). The decreased secretion of SipD, a T3SS-1 invasion protein, in the AMPR mutants may alter the ability of these cells to invade host tissue (20). Mutations in AMPR genes also caused an increase in the levels of proteins in the supernatant that are naturally found in the cell membrane (Tuf [59]), in the periplasm (OsmY, MalE, and GlpQ [60–62]), or in the cytosol (Tsf and GapA [63, 64]). The decreased secretion of flagellar proteins and the increased presence of membrane proteins in the supernatants of the Δ*virK* Δ*ybjX* mutant and the Δ*ybjX*

TABLE 6 *S.* Enteritidis deposition in eggs and colonization in ovaries[a]

Strain	No. of ovaries with positive results/total no. of ovaries (% positive) on indicated dpi		No. of eggs with positive results/total no. of eggs (% positive) on indicated dpi									Avg % positive	Total no. of eggs with positive results/total no. of eggs (% positive)
	5	10	1	2	3	4	5	6	7	8	9	10	
WT	5/6 (83.3)	2/6 (33.3)	**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)
Δ*virK*	1/6 (16.7)	2/6 (33.3)	**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)
Δ*virK* pWSK*VirK*	3/6 (50.0)	1/6 (16.7)	**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)
Δ*ybjX*	3/6 (50.0)	4/6 (66.7)	**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)
Δ*ybjX* pWSKY*bjX*	3/6 (50.0)	2/6 (33.3)	**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)
Δ*virK* Δ*ybjX*	1/6 (16.7)	2/6 (33.3)	**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)

[a] Eggs were collected at the same time daily during the infection challenge of 21-week-old laying hens with 5×10^9 CFU of *S.* Enteritidis strains. Data for eggs with positive results are in boldface. The number of hens with positive ovary colonization results is shown as the fraction of the total number of hens tested at each of two tissue collection time points. A single asterisk denotes a significant difference from the results determined for the WT strain in the average numbers of eggs with positive results on the indicated dpi (%) and in the amounts of eggs with positive results on the indicated dpi ($P < 0.05$).

mutant indicate an unstable outer membrane or membrane shearing.

A study in *S.* Typhimurium has shown that AMPR genes, through direct or indirect modification of LPS, contribute to resistance to antimicrobial peptide polymyxin B (49). We have shown in the present study not only that *virK* and *ybjX* are involved in resistance to polymyxin B but also that they are involved in resistance to AvBD-6, a host antimicrobial peptide crucial in the innate immune system (Fig. 5). Therefore, it is obvious that these AMPR genes are required for the stability of the outer membrane of *S.* Enteritidis, which in turn affects the ability of the organism to coordinate virulence functions and defend 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 (13, 49, 51). The mechanism behind this phenomenon is that genes in the PhoP/PhoQ regulon, known to be upregulated within macrophages, alter the outer membrane structure or composition for defense against the various host killing factors within these phagocytes (7, 13). We have seen that a mutation in *ybjX* is associated with decreased macrophage entry, due either to a suboptimal interaction with the macrophage or to the inability of macrophages to phagocytize the larger bacteria (Fig. 3 and 6). We have observed a survival defect of AMPR mutants inside macrophages (Fig. 6). However, we did not detect any significant defects in the ability of the mutants to colonize spleen at 5 dpi and 10 dpi (data not shown). Our data are in disagreement with findings of a previous study of *S.* Typhimurium in mice that suggested that these AMPR genes play a role in late stages of infection (49). At this time, it is not known whether the difference is serovar specific or host specific or both.

To determine the contribution of AMPR genes to intestinal colonization, we analyzed the intestinal and fecal bacterial loads. In early stages of infection, the Δ*virK* Δ*ybjX* double mutant was excreted at the lowest level (Fig. 9A), which corresponded to its inability to survive in cecal contents and its inability to withstand deoxycholic acid (Fig. 3 and 8). Complementation of *ybjX* also caused a reduced fecal bacterial load which could not be explained by the strain's ability to survive in cecal lumen or to resist deoxycholic acid. It has been previously observed that the outer core of LPS is required for the entry of *S.* Typhimurium into intestinal epithelial cells (65). However, due to experimental variations, we did not observe significant intestinal tissue colonization defects associated with *virK* and *ybjX* mutations.

S. Enteritidis is known to preferentially colonize the reproductive tract of laying hens and contaminate eggs without inducing overt clinical signs (16, 17). *S.* Enteritidis strains recovered from the field have a higher degree of heterogeneity, especially in the glucosylation in the O-chain of the LPS, than *S.* Typhimurium strains (19, 66). This heterogeneity may be caused by alternative implementations of outer membrane modification stress mechanisms in the various host environments for defense against *S.* Enteritidis (14, 17). We have seen extensive heterogeneity associated with *ybjX*. Both deletion and complementation of *ybjX* could alter the phenotypes of *S.* Enteritidis in terms of cell length, colony size, and tissue colonization. While a mutation in *virK* renders *S.* Enteritidis incapable of colonizing the ovary (Fig. 10B and Table 6), deletion of *ybjX* resulted in prolonged colonization of ovaries (Table 6) and egg contamination (Fig. 10 and Table 6). It has been previously shown that an *S.* Enteritidis (*wzz*) mutant lacking high-molecular-mass LPS (HMM-LPS) was more effective than the wild-type strain with respect to reproductive-tract colonization and egg deposition (67). It was suggested that the presence of HMM-LPS allows *S.* Enteritidis to silently colonize the chicken host by mitigating reproductive-tract responses to infection but does so without altering the incidence of egg contamination (67). A recent study identified *S.* Enteritidis LPS biosynthesis as a critical factor involved in egg white persistence (68). Our data suggest that *virK* promotes ovary colonization at the early stage of infection and that *ybjX* negatively controls late-stage ovary colonization and egg deposition. The counteractive balance may be achieved through a suppressor role of *ybjX*, as suggested by a previous study (53). This hypothesis is supported by the observation that introduction of (more than one copy of) a cloned *ybjX* gene exacerbated phenotypes.

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 structures of their outer membrane, one of the key components in the interaction between *Salmonella* and its host (21, 22). Many investigators have argued that the main difference between *S.* Typhimurium and *S.* Enteritidis with respect to survival in the chicken host is in the use of stress-induced mechanisms, including outer membrane modulation, and that this accounts for the differences seen in pandemics associated with contaminated poultry products (14, 17, 19, 46). Silent colonization of *S.* Enteritidis in chicken requires intrinsic abilities to defend against the innate immune system without inducing overt inflammation and damage (16). This report shows that AMPR genes *virK* and *ybjX* not only aid in resistance to innate antimicrobial peptides and detergents but are also involved in replication and persistence in chicken tissues and eggs. Also, the effects of AMPR genes seen in *S.* Enteritidis do not correspond to the effects seen 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.

ACKNOWLEDGMENTS

We are grateful to Larry Dangott and the staff members of the Texas A&M University Protein Chemistry Laboratory for their assistance in running the experiments using 2D SDS-PAGE gels, tandem mass spectrometry, and protein analysis, Dale Hyatt and the staff members of the Poultry Science Center for raising the hens, and the staff members of the Veterinary Medical Park and the Comparative Medicine Program and Amber Nava at Texas A&M University for all their assistance with the *in vivo* trials.

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