

Salmonella Enteritidis Strains from Poultry Exhibit Differential Responses to Acid Stress, Oxidative Stress, and Survival in the Egg Albumen

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Abstract

Salmonella Enteritidis is the major foodborne pathogen that is primarily transmitted by contaminated chicken meat and eggs. We recently demonstrated that *Salmonella* Enteritidis strains from poultry differ in their ability to invade human intestinal cells and cause disease in orally challenged mice. Here we hypothesized that the differential virulence of *Salmonella* Enteritidis strains is due to the differential fitness in the adverse environments that may be encountered during infection in the host. The responses of a panel of six *Salmonella* Enteritidis strains to acid stress, oxidative stress, survival in egg albumen, and the ability to cause infection in chickens were analyzed. This analysis allowed classification of strains into two categories, stress-sensitive and stress-resistant, with the former showing significantly ($p < 0.05$) reduced survival in acidic (gastric phase of infection) and oxidative (intestinal and systemic phase of infection) stress. Stress-sensitive strains also showed impaired intestinal colonization and systemic dissemination in orally inoculated chickens and failed to survive/grow in egg albumen. Comparative genomic hybridization microarray analysis revealed no differences at the discriminatory level of the whole gene content between stress-sensitive and stress-resistant strains. However, sequencing of *rpoS*, a stress-regulatory gene, revealed that one of the three stress-sensitive strains carried an insertion mutation in the *rpoS* resulting in truncation of σ^S . Finding that one of the stress-sensitive strains carried an easily identifiable small polymorphism within a stress-response gene suggests that the other strains may also have small polymorphisms elsewhere in the genome, which likely impact regulation of stress or virulence associated genes in some manner.

Introduction

SALMONELLA ENTERICA SEROVAR ENTERITIDIS is the most common serotype of *Salmonella* isolated from cases of foodborne gastroenteritis throughout the world (EFSA, 2007; WHO, 2008). Chickens are the single largest reservoir host for *Salmonella* Enteritidis, and source attribution studies have determined that contaminated poultry and poultry products are the major sources of human infection (Kimura *et al.*, 2004; Patrick *et al.*, 2004). In the chicken host, *Salmonella* Enteritidis is transmitted via a fecal-oral route. Young chickens <2 weeks of age often develop gastroenteritis and systemic disease with varying degrees of mortality. In contrast, most adult hens become colonized with *Salmonella* Enteritidis, but typically remain asymptomatic carriers with intermittent fecal shedding of *Salmonella*. During intestinal colonization, inflamma-

tion ensues and is characterized by an early influx of heterophils (avian counterpart to mammalian neutrophils) and macrophages that play an important role in host defense (Henderson *et al.*, 1999; Chappell *et al.*, 2009). *Salmonella* Enteritidis has the ability to survive within these cells (Henderson *et al.*, 1999; Stabler *et al.*, 1994; Okamura *et al.*, 2005) and also penetrate the mucosal epithelium, resulting in dissemination to organs, including spleen, liver, and reproductive tract (Desmidt *et al.*, 1997; Gantois *et al.*, 2009). Due to the specialized ability of *Salmonella* Enteritidis to colonize the avian reproductive tract and to contaminate internal contents of eggs, contaminated eggs or egg products have been implicated in the majority of foodborne outbreaks worldwide (Kimura *et al.*, 2004; Patrick *et al.*, 2004).

The specialized ability of *Salmonella* Enteritidis to contaminate and survive within the egg is important from the public

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health perspective. The potential for *Salmonella* Enteritidis to be deposited in egg contents primarily depends on the "virulence" of a particular strain, i.e., the ability of the bacterium to invade avian intestinal epithelium with subsequent dissemination to internal tissues (Gast and Benson, 1996; Gast, 1994; Gast and Beard, 1990). It has been reported that naturally occurring *Salmonella* Enteritidis strains vary in their virulence in chickens in terms of organ invasiveness (Gast and Benson 1995, 1996; Dhillon *et al.*, 1999), and the ability to contaminate and survive within the egg contents (Petter, 1993; Clavijo *et al.*, 2006; Yim *et al.*, 2010). These differences in virulence exist irrespective of the phage type (Poppe, *et al.*, 1993) or clonal lineages (Yim *et al.*, 2010; Olsen *et al.*, 1999). The basis for this differential virulence of *Salmonella* Enteritidis in the chicken is somewhat understood, because phenotypic variation has been traced to the level of the single nucleotide polymorphism rather than to differences in whole gene content (Morales *et al.*, 2007).

Avian innate defenses play an important role in the outcome of infection. For instance, the two major avian innate defenses that impose severe stress on an invading *Salmonella* Enteritidis include the acidic stress from gastric acidity (pH 2.6) (Joyner and Kokas, 1971; Carter and Collins, 1974) and oxidative stress from effects of hydrogen peroxide (H₂O₂) produced by infected avian heterophils and macrophages (Qureshi *et al.*, 2000; Withanage *et al.*, 2005; Kogut *et al.*, 2002). Furthermore, egg albumen contains several antibacterial substances such as lysozyme, ovotransferrin, and avian B-defensins (Van Immerseel, 2010; Kang *et al.*, 2006), thereby presenting another detrimental environment for the survival and growth of this bacterium. Assessing the ability of *Salmonella* Enteritidis strains to survive under different stress conditions encountered early during infection in chickens may facilitate better understanding of the pathogenesis of *Salmonella* Enteritidis. Nevertheless, limited studies have been conducted to determine the responses of *Salmonella* Enteritidis to various stress conditions (Yim *et al.*, 2010; Humphrey *et al.*, 1993, 1995). For instance, Humphrey *et al.* (1995) reported that *Salmonella* Enteritidis strains isolated from poultry had reduced tolerance to acid and oxidative stress as compared with the strains isolated from human clinical cases. In another study, Yim *et al.* (2010) reported that vast majority of *Salmonella* Enteritidis strains isolated from food sources showed diminished survival in egg albumen as compared with the clinical strains. In addition, *Salmonella* Enteritidis produces a specialized capsular-like lipopolysaccharide O-antigen that contributes to the survival of the pathogen in the egg, but its production varies between strains (Guard-Bouldin *et al.*, 2004). We hypothesized that differential virulence of *Salmonella* Enteritidis strains is due to the differential fitness in the stressful environments encountered in the host during infection. To address this hypothesis, we tested the ability of several *Salmonella* Enteritidis strains to survive under various stressful conditions that may be encountered in the chicken host such as acidic-stress, oxidative-stress, growth in egg albumen, and *in vivo* virulence in orally inoculated chickens.

Methods

Bacterial strains

A panel of six *Salmonella* Enteritidis strains (UK, G1, BC8, C19, C45, and G45) isolated from poultry or poultry-

TABLE 1. PHENOTYPIC AND GENOTYPIC CHARACTERISTICS OF POULTRY-ASSOCIATED *SALMONELLA* ENTERITIDIS STRAINS USED IN THIS STUDY (SHAH *ET AL.*, 2011)

Strain	Phage type	MLVA type	Caco-2 cell invasiveness	Survival in chicken macrophages	Motility	Biofilm
C19	PT13	11	Low	Low	Low	Negative
C45	PT13	11	Low	Low	Low	Negative
BC8	PT8	9a	High	High	High	Mixed
G1	PT4	13a	Medium	High	High	Positive
G45	PT13a	4a	Low	Low	Low	Negative
UK	PT4	13a	High	High	High	Positive

MLVA, multi-locus variable-tandem repeat analysis.

associated environments were analyzed in this study. Previous work from our laboratory showed that the UK (corresponds to the sequenced phage type-4 P125109 strain), G1 (phage type-4), and BC8 (phage type-8) strains differ from C19 (phage type-13), C45 (phage type-13), and G45 (phage type-13a) strains in terms of their invasiveness into human intestinal epithelial cells (Caco-2), virulence in orally challenged mice, motility, biofilm production, and phage types (Table 1) (Shah *et al.*, 2011). Frozen stocks of cultures were grown on Luria-Bertani (LB) agar incubated at 37°C for overnight. For all the experiments, a single colony from the overnight culture was inoculated into LB broth and grown at 37°C or 42°C for 16 h with shaking at 200 rpm.

Survival assays

The stationary phase (16 h) cultures of *Salmonella* Enteritidis strains were used to measure their survival rates in LB broth adjusted to pH 2.6 ± 0.02 with 1 M HCl (Humphrey *et al.*, 1993, 1995) and in normal saline (0.9% NaCl) after addition of H₂O₂ to a final concentration of 15 mM (Robbe-Saule *et al.*, 2003). Controls included the cultures incubated in the same media but in the absence of stressors. Stationary phase cultures of *Salmonella* Enteritidis strains were used because this growth phase provides a reliable model to measure the differences in tolerance to acid and oxidative stress (Humphrey *et al.*, 1995). All the media, tubes and reagents were prewarmed to avoid a sudden shift in temperatures that may otherwise markedly alter stress tolerance in *Salmonella* Enteritidis (Humphrey *et al.*, 1993). For the acid stress survival assay, ~1 × 10⁸ colony forming units (CFU) of each *Salmonella* Enteritidis strain was inoculated in LB (pH 2.6) at 37°C or at 42°C followed by incubation at respective temperatures. For the oxidative stress survival assay, ~1 × 10⁸ CFU of the stationary phase cultures were resuspended in normal saline at either 37°C or 42°C. Subsequently, H₂O₂ was added to the final concentration of 15 mM, mixed thoroughly, and the suspension was incubated at 37°C or at 42°C. Aliquots from acid and oxidative stress cultures were collected at 10 min and 1 h post-inoculation. Serial 10-fold dilutions of each culture was prepared using maximum recovery diluent (MRD; Oxoid, Lenexa, KS) and plated on LB agar for enumeration of viable CFU. Each strain was tested in duplicate, and at least three independent experiments were completed.

Growth in egg albumen

The ability of *Salmonella* Enteritidis strains to survive in egg albumen was quantified as described previously with minor

modifications (Lu *et al.*, 2003). Briefly, organic, unfertilized, antibiotic-free eggs were purchased from Chino Valley Ranchers, CA and stored for less than 1 week at 4°C. For each experiment, two to three eggs were disinfected by immersion into 70% ethanol and aseptically broken to collect egg albumen into a sterile container. Egg albumen was pooled, and 1 mL aliquots were distributed into 96-well blocks (Qiagen, Valencia, CA). An overnight culture of each bacterial strain was added to egg albumen to a final concentration of ~500 CFU/mL and thoroughly mixed and incubated at 25 ± 2°C for 24 h. After incubation, the bacteria-albumen mixture was diluted in MRD and plated on LB agar to enumerate the viable bacterial counts. Uninoculated egg albumen served as a negative control. Five independent experiments were completed for each strain.

Comparative genomic hybridizations

The microarray was constructed by MYcroarray Inc. (Ann Arbor, MI) using the genomic information for the sequenced *Salmonella* Enteritidis PT4 P125109 (Thomson *et al.*, 2008) which is referred to as the UK strain in this study. This microarray consists of a total of 16,320 features. Of these, 4,343 are control features (spike-in positive controls, empty spots, negative control probes, etc). The remaining 11,977 features (~47-mers) represent 4,200 ORFs as follows: three specific probes/ORF (3,742 genes), two specific probes/ORF (293 genes), and one specific probe/ORF (165 genes). Three arrays per slide were generated by *in situ* synthesis using a proprietary light-directed oligonucleotide synthesis technology (www.mycroarray.com). Each strain was tested in triplicate (3 arrays/strain) using hybridization protocols as described previously (Call *et al.*, 2003). Slides were scanned using a Genepix400B scanner (Axon Instruments, Inverurie, Scotland). After acquisition of signal intensity data, each spot was corrected by subtracting local background values and each "corrected" spot intensity was used to calculate median signal intensity for each array. Inferior spot intensities (empty spots or those where signal intensity was less than two times the background) were reset to an arbitrary number (50) and were not used in the median calculation. The array median intensity was then used for within array normalization. The data was log₂ transformed and quantile normalized between arrays using SOLO (<http://www-microarrays.u-strasbg.fr/Solo/index.html>). Finally, the estimated probability of presence (EPP) for each probe was determined using a GACK-transformation as described previously (Kim *et al.*, 2002). Microarray data and platform information have been submitted to GEO (under accession no. GSE33102).

rpoS gene sequencing

The total genomic DNA from each *Salmonella* Enteritidis strain was extracted using DNeasy Tissue kit (Qiagen) according to the manufacturer's protocol. The full length *rpoS* gene (993 bp) was amplified from all six strains using primers *rpoS*_F (5'-atgagtcagaatacgtgaaag-3') and *rpoS*_R (5'-ttactcgcggaacagcgc-3'). PCR amplification included 28 cycles, each consisting of 30 s of denaturation at 96°C, 30 s of annealing at 50°C, and 45 s of extension at 72°C. PCR products were cloned into pGEM®-T Easy vector (Promega, Madison, WI) according to manufacturer's instructions. The *rpoS* gene insert was sequenced using universal reverse

(5'-actttatgccggctcgatgtt-3') and a universal forward (5'-atgtgtgcaaggcgattaagttggg-3') primer at the Washington State University Genomics Core. The *rpoS* sequences were aligned against *rpoS* gene from a reference UK strain P125109 (GenBank accession no. AM933172) (Thomson *et al.*, 2008). Sequence alignment and single nucleotide polymorphism were detected using Geneious Pro 5.3 (Biomatters Ltd., Auckland, New Zealand). The sequences of *rpoS* genes were deposited in the GenBank under the following accession numbers: JN588998 (UK), JN588996 (G1), JN588997 (BC8), JN588999 (C19), JN589000 (C45), and JN588995 (G45).

Chicken virulence assay

Twenty-one 1-day-old chickens were obtained from Belt Hatchery (Fresno, CA) and distributed in seven groups ($n = 3$ /group) in environmentally controlled isolation cages. Cloacal swabs were taken prior to placement of chicks in cages and examined for the presence of *Salmonella* by selective enrichment in tetrathionate broth (TTB; Difco, Franklin Lakes, NJ) followed by plating onto XLD agar (Difco). Antibiotic-free flock raiser diet (Purina, St. Louis, MO) and water was provided *ad libitum* throughout the experimental period. At 3 days of age, chickens were challenged orally with stationary phase cultures of *Salmonella* Enteritidis strains at a concentration of ~10⁷ CFU. One group served as the uninoculated negative control. The chickens in all groups were euthanized at 4 days post-infection, necropsied, and the small intestine, ceca, liver, and spleen were examined by selective enrichment in TTB followed by plating onto XLD-agar. The viable counts of *Salmonella* per gram in liver and spleen tissues from all the chickens were obtained by emulsification of weighed tissues in PBS followed by plating of serial dilutions of the ground tissues on XLD-agar plates. Presumptive *Salmonella* colonies were confirmed by slide agglutination test using serogroup D antiserum (Difco). The animal challenge experiments were conducted in accordance with the protocol approved by the Washington State University Institutional Animal Care and Use Committee (WSU IACUC).

Statistical analysis

Data was analyzed using one-factor analysis of variance (ANOVA) with Tukey's Kramer post-test in NCSS 2007 (NCSS, LLC, Kaysville, UT).

Results and Discussion

Because the ability of *Salmonella* to withstand stomach acidity is a recognized virulence attribute, we tested the survivability of stationary phase cultures of six *Salmonella* Enteritidis strains by substantially mimicking the avian stomach environment (pH 2.6) (Joyner and Kokas, 1971). While there were no differences in the survival of *Salmonella* Enteritidis strains in the absence of acid-stress (data not shown), significant differences in the tolerance of *Salmonella* Enteritidis strains to acid-stress were observed (Table 2). When 8 log CFU of *Salmonella* Enteritidis strains were exposed to pH 2.6 at 37°C for 10 min, the survival rate of UK, G1, and BC8 strains (>7 log CFU) was significantly higher ($p < 0.05$, Tukey's Kramer test) as compared with the C19, C45, and G45 strains (<5 log CFU) (Table 2). At 1 h post-incubation, <1 log CFU of C19 and C45 and 1.2 log CFU of G45 strain survived, whereas

TABLE 2. SURVIVAL VALUES FOR HIGH- AND LOW-PATHOGENIC *SALMONELLA* ENTERITIDIS STRAINS EXPOSED TO ACIDIC (pH 2.6) AND OXIDATIVE (15 mM H₂O₂) STRESS

Conditions	Number of survivors (mean log ₁₀ CFU ± SE) ^a					
	Stress-resistant			Stress-sensitive		
	UK	BC8	G1	C19	G45	C45
pH 2.6 for 10 min (37°C)	7.33 ± 0.4	7.08 ± 0.43	7.33 ± 0.36	4.05 ± 1.01 ^b	4.84 ± 0.75 ^b	4.14 ± 1.06 ^b
pH 2.6 for 1 h (37°C)	1.96 ± 1.15	2.88 ± 1.67	3.21 ± 1.35	<0.01 ^b	1.2 ± 0.41 ^b	0.09 ± 0.09 ^b
pH 2.6 for 10 min (42°C)	5.35 ± 0.9	4.28 ± 1.29	5.02 ± 1.17	0.66 ± 0.5 ^b	1.02 ± 0.65 ^b	1.01 ± 0.64 ^b
pH 2.6 for 1 h (42°C)	0	0	0	0	0	0
15 mM H ₂ O ₂ for 10 min (37°C)	8.07 ± 0.04	8.07 ± 0.06	8.06 ± 0.05	6.1 ± 0.18 ^b	5.53 ± 0.19 ^b	6.33 ± 0.19 ^b
15 mM H ₂ O ₂ for 1 h (37°C)	7.95 ± 0.05	7.81 ± 0.24	7.92 ± 0.12	1.54 ± 1.04 ^b	0.4 ± 0.4 ^b	1.47 ± 0.6 ^b
15 mM H ₂ O ₂ for 10 min (42°C)	8.06 ± 0.08	8.08 ± 0.1	8.06 ± 0.07	6.4 ± 0.23 ^b	5.33 ± 0.19 ^b	6.5 ± 0.07 ^b
15 mM H ₂ O ₂ for 1 h (42°C)	7.94 ± 0.09	7.43 ± 0.19	7.85 ± 0.01	0	0	0

^aAn initial 8 log colony forming units (CFU) of each strain was used in each stress-survival assay and the mean log₁₀ CFU ± standard error (SE) were calculated from three independent experiments.

^bFor each stressor, means followed by asterisk indicate a significant difference from stress-resistant strains exposed to same stressor ($p < 0.05$, Tukey's Kramer test).

>1.96 log CFU of the UK, G1, and BC8 strains survived this treatment. These differences were statistically significant ($p < 0.05$). At 42°C (chicken body temperature), the counts of C19, C45, and G45 strains declined from an initial 8 log CFU to ≤1 log within 10 min. Thus, killing of stress-sensitive strains was more pronounced at the higher body temperature of the bird (Table 2). In contrast, >4 log CFU of the UK, G1, and BC8 strains survived this treatment, indicating that these strains were resistant to short-term acidic stress at 37°C and 42°C. We were not able to recover any of the *Salmonella* Enteritidis strains after 1 h of incubation at 42°C, suggesting that none of the strains survived prolonged acidic stress at high temperature. These results indicate that C19, C45, and G45 strains are acid-sensitive, whereas the UK, G1, and BC8 strains are relatively acid-resistant (Table 2).

Hydrogen peroxide produced by avian macrophages and heterophils plays a crucial role in *Salmonella* killing because it can penetrate cell membranes and may act on intracellular targets (Desmidt *et al.*, 1997; Qureshi *et al.*, 2000; Withanage *et al.*, 2005; Kogut *et al.*, 2002). Therefore, we determined the ability of *Salmonella* Enteritidis strains to counteract the effects of hydrogen peroxide. Irrespective of the temperature (37°C or 42°C) or time (10 min or 1 h) of incubation, there was no significant decline in the counts of UK, G1, and BC8 strains (Table 2) indicating that these strains were highly resistant to the oxidative stress induced by hydrogen peroxide. In contrast, the counts of C19, C45, and G45 strains declined by 1.5–2.5 log within 10 min at 37°C or 42°C (Table 2). By 1 h, the counts further declined by 6.5–7.5 log, indicating that these strains were highly sensitive to the oxidative stress induced by hydrogen peroxide (Table 2). Overall, these results indicate that C19, C45, and G45 strains (stress-sensitive strains) show impaired ability to survive in both acid and oxidative stress when compared with the UK, G1, and BC8 strains (stress-resistant strains).

To assess the virulence (i.e., the ability to invade and systemically spread in the internal organs) of stress-sensitive and stress-resistant *Salmonella* Enteritidis strains in chickens, 3-day-old chickens were orally inoculated with ~10⁷ CFU of each strain and sacrificed at 4 days post-infection. For stress-resistant strains, the mean log₁₀ CFU of *Salmonella* Enteritidis/

gram of liver ranged from 1.57 ± 0.79 (UK) to 1.75 ± 0.14 (G1), whereas mean log₁₀ CFU of *Salmonella* Enteritidis/gram of spleen ranged from 3.22 ± 0.32 (BC8) to 3.96 ± 0.05 (UK) (Fig. 1). In contrast, none of the stress-sensitive strains were recovered from either liver or spleen by either direct plating or by enrichment procedure, indicating that the acid-sensitive strains had impaired ability to invade the internal tissues. Similar to the results obtained in this study, Jorgensen *et al.*, (2000) reported that two acid-sensitive *Salmonella* Typhimurium strains failed to invade chicken tissues, whereas Humphrey *et al.* (1996) reported that one acid and H₂O₂ sensitive *Salmonella* Enteritidis strain was significantly less invasive in chickens when compared with one acid and H₂O₂ resistant strain. It was also reported that the fecal carriage rates of stress-sensitive strains of *Salmonella* Typhimurium were not significantly different from those of stress-resistant strains (Jorgensen *et al.*, 2000; Williams *et al.*, 1998). In this study, the intestinal samples collected from chickens challenged with

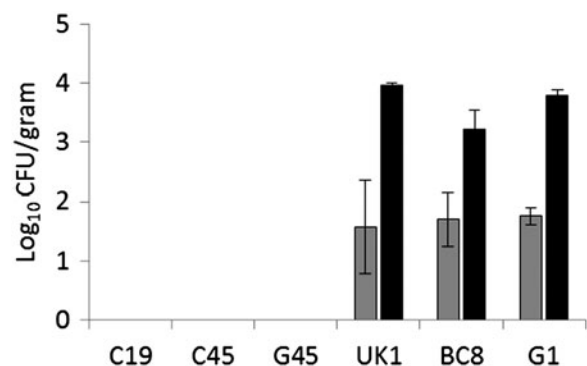


FIG. 1. The mean log₁₀ colony forming units (CFU) ± standard error (SE) of stress-sensitive and stress-resistant *Salmonella* Enteritidis strains recovered from liver (gray bars) and spleen (black bars) of chicks. Chicks in each group ($n = 3$) were orally inoculated at number (#) of days of age with ~1 × 10⁷ CFU of *Salmonella* Enteritidis strains, and the challenge strains were recovered from the internal organs at 4 days post-infection.

stress-resistant strains were positive for *Salmonella* by selective enrichment procedure, indicating that intestinal carriage of these strains was not affected. Nevertheless, none of the intestinal samples collected from chickens infected with stress-sensitive strain were positive for *Salmonella*, indicating that stress-sensitive strains had an impaired ability to colonize the intestine. Studies on oral infection of *Salmonella* Enteritidis in mice have shown that only 1% of the inoculum survives the low pH during the passage through the stomach (Carter and Collins, 1974). About 80% of the bacteria that survive the passage through the stomach are passed with the feces within 6–10 h post-infection, whereas 15% remain localized in the lumen of cecum and large intestine, while only 5% manage to penetrate the intestinal wall of the small intestine and reach gut associated lymphoid tissues (Carter and Collins, 1974). Because the stress-sensitive strains tested in this study showed impaired ability to survive in gastric acidity and oxidative stress, these strains appear to have cleared from the intestine before the birds were sacrificed 4 days post-infection. Additional time-course studies to investigate changes in the intestinal carriage and systemic spread of stress-sensitive and stress-resistant strains may be required to fully understand the differences in their kinetics of infection in chickens.

It has been hypothesized that stress-induced survival mechanisms may enable certain *Salmonella* Enteritidis strains to cope with the antimicrobial compounds present in the egg albumen (Van Immerseel, 2010). Consequently, we tested all six *Salmonella* Enteritidis strains for their ability to survive and/or grow in egg albumen. With the starting inoculum of ~500 CFU, all the stress-resistant strains grew in egg albumen up to 4–5 log after 24 h of incubation at 25°C (Fig. 2). In contrast, two stress-sensitive strains (C19 and C45) did not survive or grow in egg albumen, whereas one strain (G45) grew as well as the stress-resistant strains. These results indicate that some stress-sensitive strains may also have an impaired ability to respond to the hostile environment of egg albumen—an environment that includes iron restriction, high pH, and other enzymatic activities. Further research is required to see if additional factors, such as expression of an O-antigen capsule, also contribute to the survival and growth of some strains *Salmonella* Enteritidis in albumen.

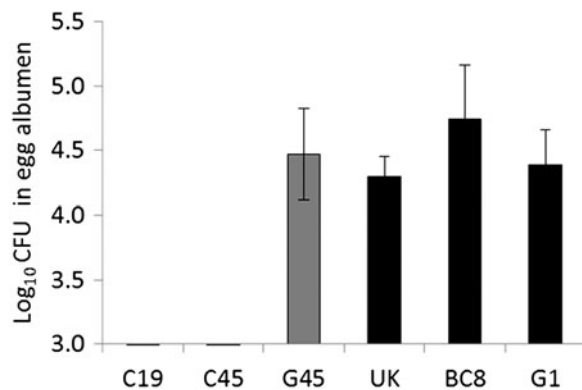


FIG. 2. The mean log₁₀ colony forming units (CFU) ± standard error (SE) of stress-sensitive (gray bars) and stress-resistant (black bars) *Salmonella* Enteritidis strains in egg albumen. An overnight culture of each bacterial strain was added to egg albumen to a final concentration of ~500 CFU/mL and incubated at 25 ± 2°C for 24 h.

To determine if the differential stress response and virulence observed in this study could be due to the differences in the gene content between different strains, we compared the genomes of all the strains using CGH microarray designed based on the genomic information available for the sequenced strain. The CGH analysis revealed no consistent differences in the genomes between the six strains tested in this study. These results corroborate previous reports indicating that *Salmonella* Enteritidis strains are relatively genetically homogeneous, despite geographical, temporal, and source differences between the different strains (Porwollik *et al.*, 2005; Morales *et al.*, 2005; Olson *et al.*, 2007; Betancor *et al.*, 2009). It is important to point out that the microarray used in this study contained genes representing a single *Salmonella* Enteritidis strain and therefore only genes that are present on the array can be assayed. Some strains tested in this study may contain additional genes (e.g., phage-related genes) that were not represented on the microarray. Overall, these results indicate that the differential stress-response and virulence is less likely due to the variation in the whole gene content, but are more likely due to small polymorphisms that may alter regulation or expression of stress or virulence associated genes. To determine if the phenotypic differences were related to the *rpoS* gene, we sequenced the full length *rpoS* from all the six *Salmonella* Enteritidis strains. Several studies have shown that σ^S , encoded by the *rpoS* gene, controls the general stress response of bacteria, in particular the ability to resist hydrogen peroxide and acid stress during stationary phase (Hengge-Aronis, 2002). In addition, it has been reported that RpoS contributes to the virulence of *Salmonella* (Jorgensen *et al.*, 2000; Nickerson and Curtiss, 1997). The *rpoS* gene in two stress-resistant strains (UK and G1) and one stress-sensitive strain (C19) was 100% identical in DNA sequence to the *rpoS* gene of *Salmonella* Enteritidis P125109 (GenBank accession no. AM933172). Strains BC8 contained a synonymous substitution from G to A at nucleotide 375 and a non-synonymous mutation at codon 36 (AGT [Ser] to AGG [Arg]). Strain C45 contained a non-synonymous mutation at codon 126 (GGG [Gly] to GAG [Glu]). Strain G45 contained two mutations, a synonymous substitution from T to C at nucleotide 605 and an insertion of A at nucleotide position 666. The insertion resulted in the introduction of an internal stop codon (TAA). Thus, one of the three stress-sensitive strains analyzed had a discernible mutation that interrupted the ORF of *rpoS*, consistent with the hypothesis that differential survivorship between strains is related to the single nucleotide polymorphisms in the chromosome. It is possible that the other strains may also have small polymorphisms elsewhere in the genome, which likely impact regulation of stress or virulence associated genes in some manner.

To determine whether any of the mutations altered the production of σ^S , we measured the activity of *rpoS* indirectly by analysis of hydrogen peroxidase II (HPII) activity using a semiquantitative catalase test as described previously (Taylor and Achanzar, 1972). Catalase test is based on principle that when 3% H₂O₂ is added to the bacterial colonies grown on LB plates, HPII breaks down H₂O₂ with the concomitant release of O₂ resulting in bubbling of the colony. Strains producing RpoS bubble vigorously (+++), whereas strains bearing null alleles either do not form bubbles (-) or bubble only slightly (+) (Taylor and Achanzar 1972). We found that UK, G1, BC8, C19 and C45 strains formed vigorous bubbles (+++),

suggesting that *rpoS* mutations in strains BC8 and C45 did not affect the σ^S function. In contrast, G45 strain with truncated *rpoS* did not form bubbles (-), indicating impaired σ^S function and a possibility of impaired KatN, an *rpoS* regulated non-hem catalase expression (Robbe-Saule, *et al.*, 2001). It is important to note that the G45 strain is impaired in motility and also lacks a large virulence plasmid (Shah *et al.*, 2011) and *Salmonella* plasmid virulence (*spv*) genes whose expression is controlled by *rpoS* during systemic infection (Jorgensen *et al.*, 2000; Nickerson and Curtiss, 1997). Therefore it is likely that the impaired motility or regulation of virulence genes in this particular strain may have led to the reduced pathogenicity in chickens. Interestingly, the growth of G45 strain in the egg albumen was not affected indicating that σ^S may not be required for the survival of *Salmonella* Enteritidis in egg albumen (Fig. 2). Previous studies have reported that genes involved in bacterial cell wall structure and function, amino acid and nucleic acid metabolism, motility and stress responses may contribute to the survival of *Salmonella* Enteritidis in egg albumen (Clavijo *et al.*, 2006; Lu *et al.*, 2003; Gantois *et al.*, 2008).

Conclusion

The results of this study indicate that wild-type strains of *Salmonella* Enteritidis show differential response to the acid stress, oxidative stress and survival in the egg albumen. Stress-sensitive strains may be less virulent as compared to the stress-resistant strains and therefore differential virulence of wild-type strains may be attributed to the underlying differences in their response to common stressful conditions encountered during infection. The differences in the regulation of virulence or stress-associated genes contribute to, but do not fully explain, the phenotypic differences observed in this study. Comparative transcriptomic and proteome profiling of these well-characterized stress-sensitive and stress-resistant strains may provide further insights into the molecular basis of differential stress response and virulence of *Salmonella* Enteritidis strains.

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Disclosure Statement

No competing financial interests exist.

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