

## Identification of Genes Associated with Survival of *Salmonella enterica* Serovar Enteritidis in Chicken Egg Albumen

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*Salmonella enterica* consists of over 2,000 serovars that are major causes of morbidity and mortality associated with contaminated food. Despite similarities among serovars of *Salmonella enterica*, many demonstrate unique host specificities, epidemiological characteristics, and clinical manifestations. One of the unique epidemiological characteristics of the serovar Enteritidis is that it is the only bacterium routinely transmitted to humans through intact chicken eggs. Therefore, *Salmonella enterica* serovar Enteritidis must be able to persist inside chicken eggs to be transmitted to humans, and its survival in egg is important for its transmission to the human population. The ability of *Salmonella enterica* serovar Enteritidis to survive in and transmit through eggs may have contributed to its drastically increased prevalence in the 1980s and 1990s. In the present study, using transposon-mediated mutagenesis, we have identified genes important for the association of *Salmonella enterica* serovar Enteritidis with chicken eggs. Our results indicate that genes involved in cell wall structural and functional integrity, and nucleic acid and amino acid metabolism are important for *Salmonella enterica* serovar Enteritidis to persist in egg albumen. Two regions unique to *Salmonella enterica* serovar Enteritidis were also identified, one of which enhanced the survival of a *Salmonella enterica* serovar Typhimurium isolate in egg albumen. The implication of our results to the serovar specificity of *Salmonella enterica* is also explored in the present study.

Salmonellosis is one of the leading causes of food-borne diseases in the United States and throughout the world (10, 44, 56; <http://www.who.int/salmsurv/en/>). The causative agent for salmonellosis, *Salmonella enterica*, can be further classified into over 2,000 serovars (6). Among the *Salmonella enterica* serovars, the complete genomes of five serovars Choleraesuis, Enteritidis, Paratyphi, Typhi, and Typhimurium have been determined (13, 40, 41, 45; <http://www.ncbi.nih.gov>; <http://www.sanger.ac.uk/Projects/Microbes/>). These serovars of *Salmonella enterica* share remarkable similarities in both linear organization and sequence of the genomes, with sequence homologies of the conserved regions ranging from 96 to 99% (16). However, each serovar can differ significantly from another serovar in host specificity, as well as clinical and epidemiological characteristics. For example, serovar Typhi only infects humans, whereas serovars Typhimurium and Enteritidis infect a wide range of hosts including humans, rodents, and poultry (41, 45). Serovars of *Salmonella enterica* also display distinct routes of transmission. Both serovars Typhimurium and Enteritidis infect poultry; however, serovar Typhimurium is more likely to be transmitted to humans through chicken meat, whereas serovar Enteritidis is mostly transmitted to humans through chicken eggs (5, 24, 32). The molecular basis that underlies the serovar specificity is poorly understood.

The two leading *Salmonella enterica* serovars that cause salmonellosis in the United States are *Salmonella enterica* serovar Typhimurium (22% of all *Salmonella* infections) and Enteritidis (17% of all *Salmonella* infections) (6). In the mid 1990s,

*Salmonella enterica* serovar Enteritidis temporarily surpassed *Salmonella enterica* serovar Typhimurium as the primary cause of salmonellosis and is considered an emerging bacterial pathogen (59). *Salmonella enterica* serovar Enteritidis is the only human pathogen routinely found in intact chicken eggs, although other serovars of *Salmonella* are often found in a poultry farm environment and may contaminate eggs when egg shells are cracked (5, 24, 26, 43). It has been shown that both *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis infect the reproductive organs of chickens and contaminate forming eggs (internal eggs before they are laid). However, only *Salmonella enterica* serovar Enteritidis persists after eggs are laid (31, 32), and contaminated eggs may then transmit *Salmonella enterica* serovar Enteritidis to humans through the consumption of raw or undercooked eggs. Therefore, the contamination and persistence of *Salmonella enterica* serovar Enteritidis in chicken eggs represent a unique epidemiological characteristic of this bacterium that is essential for its eventual transmission to humans.

Little is known about the bacterial factors that allow *Salmonella enterica* serovar Enteritidis to survive in eggs and contribute to its epidemiological association with chicken eggs. In contaminated eggs, *Salmonella enterica* serovar Enteritidis can be deposited into both albumen and yolk. It is more frequently deposited into the albumen, especially in naturally contaminated eggs (8, 27, 52). We and others have shown that egg albumen can control the proliferation of *Salmonella enterica* serovar Enteritidis (1, 21, 22) and is bactericidal toward *Salmonella enterica* serovar Enteritidis when the inoculum is low (37). However, it is unclear how egg albumen controls bacteria and what constitutes its antimicrobial activities. We have previously reported that genes *yafD* and *xthA* are necessary for

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*Salmonella enterica* serovar Enteritidis to survive in egg albumen, possibly by repairing DNA damage caused by egg albumen (37). In the present study, we sought to determine the molecular basis of the survival of *Salmonella enterica* serovar Enteritidis in chicken egg albumen by systematically identifying genes necessary for this survival using transposon mutagenesis and mutant library screening approach. Here we report the identification of *Salmonella enterica* serovar Enteritidis gene families and functional systems that are responsible for its persistence in egg albumen and discuss the implications of our findings to the interaction of *Salmonella enterica* serovar Enteritidis with egg albumen and the serovar specificity of *Salmonella enterica*.

#### MATERIALS AND METHODS

**Bacterial strains and plasmids.** All bacterial strains used in this analysis are listed in Table 1. Isolates of *S. enterica* serovar Enteritidis and Typhimurium used in this analysis are clinical isolates, whereas both animal isolates and laboratory strains of *Escherichia coli* were used in this analysis (Table 1). Bacteriophage P22 was used for generalized transduction (39). All bacterial strains were grown in Luria-Bertani (LB) broth (Difco, Sparks, MD) at 37°C with shaking. Antibiotics were added as appropriate.

**Quantification of survival of bacteria in egg albumen.** The ability of different bacterial strains to survive in egg albumen was quantified as described previously (37). Organic, antibiotic-free eggs from a local farm were bought and stored at 4°C for up to 1 week until use. Egg albumen from two to eight eggs was pooled and beaten with an electric mixer for 3 min at the lowest speed. All bacterial strains were grown in LB broth overnight at 37°C with shaking before being used for assays. Antibiotics were added when appropriate. An overnight culture of bacteria was added to 2 ml of albumen in an Eppendorf tube to a final concentration of  $1 \times 10^3$  to  $2 \times 10^3$  CFU/ml and thoroughly mixed. The tubes were incubated at 37°C, and aliquots of the bacteria and egg albumen mix were plated on LB agar plates at the beginning and after different periods of incubation. Surviving bacteria were enumerated after overnight incubation. Each assay was repeated at least three times.

**Construction of transposon (Tn) mutant library of *Salmonella enterica* serovar Enteritidis.** A transposon mutant library of *Salmonella enterica* serovar Enteritidis SE2472 was constructed by using the EZ::Tn system by Epicentre (Madison, WI). The kanamycin resistance gene was PCR amplified from plasmid pKD4 (12) with the primers 5'-AAGTGAAGATTCGTTAGGCTGGAGCTGCTC-3' and 5'-CCACATGAATTCCATATGAATATCCTCCTTAG-3'. The PCR products were digested with EcoRI and cloned into the EcoRI site of the vector pMOD3 (Epicentre, Madison, WI). The resulting plasmid contains a kanamycin resistance cassette and is referred to as pMOD3-Kan (see Fig. 2). Transposon DNA was amplified from the pMOD3-Kan plasmid DNA by PCR with primers 5'-GTCAGTGAGCGAGGAAGCGGAAG-3' and 5'-ATTCAGGCTGCGCAA CTGT-3', digested with PshAI to generate phosphorylated ends, and used to assemble transposomes according to the manufacturer's instructions (Epicentre). The assembled transposomes were then electroporated into SE2472 for transposition to occur, and bacteria carrying transposons were selected for resistance to kanamycin. By using this method, a transposon library of approximately 3,000 independent colonies was constructed. It is generally believed that insertions by EZ::Tn are random. To confirm this, we randomly selected 15 colonies and prepared genomic DNA for southern hybridization. The genomic DNA was digested with XhoI and hybridized with a probe for Kan<sup>r</sup>. The DNA fragments containing the Kan<sup>r</sup> are of different sizes, a finding consistent with the notion that the Tn insertions are likely to be random (data not shown). Subsequent identification of Tn insertion sites in the mutants characterized in the present study also showed that the insertion sites are widely distributed in the genome.

**Screening the *Salmonella enterica* serovar Enteritidis SE2472 Tn mutant library for ES mutants.** Egg albumen was prepared as described above for the quantification of survival of bacteria in egg albumen. Two hundred microliters of egg albumen was placed into each well of a 96-well plate, and an overnight culture of bacteria was diluted to a concentration of approximately  $3 \times 10^3$  CFU/ml in the egg albumen and thoroughly mixed. Ten-microliter aliquots from each well were plated on LB agar plates immediately after the addition of bacteria to egg albumen (0-h samples) and after incubation at 37°C for 24 h (24-h samples). The bacterial concentration after 24 h of incubation was calculated and compared to the concentration at 0 h. Mutants that demonstrated a significant

TABLE 1. Bacterial strains<sup>a</sup>

Bacterial strain	Characteristics	Source or reference
<i>E. coli</i>		
DH5 $\alpha$	F <sup>-</sup> $\phi$ 80dlacZ $\Delta$ M15 $\Delta$ (lacZYA-argF)U169 deoR recA1 endA1 hsdR17(r <sub>K</sub> <sup>-</sup> m <sub>K</sub> <sup>+</sup> ) phoA supE44 $\lambda$ <sup>-</sup> thi-1 gyrA96 relA1	Gibco-BRL
HB101	F <sup>-</sup> mcrB mrr hsdS20(r <sub>B</sub> <sup>-</sup> m <sub>B</sub> <sup>-</sup> ) recA13 leu ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20(Sm <sup>r</sup> ) supE441	Sigma-Aldrich
MG1665	Wild-type <i>E. coli</i> K-12	
KSU-9	Animal isolate of <i>E. coli</i>	34
KSU-12	Animal isolate of <i>E. coli</i>	34
<i>S. enterica</i> serovar Enteritidis		
SE2472	Clinical isolate, phage type 4	38
SE6782	Clinical isolate, phage type 4	38
SE8464	Clinical isolate, phage type 4	38
SE8743	Clinical isolate, phage type 4	38
SE10871	Clinical isolate, phage type 4	38
SE4052	Clinical isolate, phage type 8	38
SE4081	Clinical isolate, phage type 8	38
SE4191	Clinical isolate, phage type 8	38
SE4241	Clinical isolate, phage type 8	38
SE4386	Clinical isolate, phage type 8	38
SE2107	Clinical isolate, RDNC	This study
SE2606	Clinical isolate, phage type 8	This study
SE0052	Clinical isolate, phage type 13	This study
SE0718	Clinical isolate, phage type 4	This study
SE0430	Clinical isolate, phage type 4	This study
<i>S. enterica</i> serovar Typhimurium		
ST3665	Clinical isolate	This study
ST3744	Clinical isolate	37
ST3964	Clinical isolate	This study
ST3864	Clinical isolate	This study
ST10428	Clinical isolate	This study
ST2258	Clinical isolate	This study
ST2297	Clinical isolate	This study
ST2298	Clinical isolate	This study
ST2302	Clinical isolate	This study
ST2327	Clinical isolate	This study

<sup>a</sup> The *E. coli*, *Salmonella enterica* serovar Enteritidis, and *Salmonella enterica* serovar Typhimurium strains used in this study are listed. The genotype of each bacterial strain is listed when known; otherwise, the source of the bacterial strain is listed. For *Salmonella enterica* serovar Enteritidis isolates, the phage type for each strain is listed. RDNC, reacts but does not conform.

decrease in survival compared to the wild-type SE2472 were selected and designated egg-susceptible (ES) mutants. Subsequently, the Tn insertions in the ES mutants from the initial screening were transduced into fresh SE2472, and their susceptibility to egg albumen was retested at least three times. ES mutants that consistently demonstrated decreased survival in egg albumen were chosen for further analysis.

**Identification of Tn insertion sites.** The transposon insertion sites in the Tn mutants were identified either by a three-step PCR or a rescue cloning procedure (15). For the three-step PCR procedure, a single primer K4 (5'-GATCTCATG CTGGAGTTC-3') was used for all PCRs. Briefly, 1 ml of overnight bacterial culture in LB was spun down and resuspended in 50  $\mu$ l of water. A bacterial lysate was prepared by boiling the resuspended bacteria for 10 min and spinning at maximum speed in a microcentrifuge for 15 min. One microliter of the lysate prepared from the Tn mutants was first amplified at 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s (35 cycles). The PCR product after the first round of amplification was ethanol precipitated and digested with DpnI (New England Biolabs, Beverly, MA). After digestion, 1  $\mu$ l of digestion mix was used as a template for

a second-round PCR amplification (94°C for 30 s, 30°C for 30 s, and 72°C for 45 s [35 cycles]). The PCR products were further amplified at the same conditions as the first-round PCR, purified with a PCR purification kit (QIAGEN, Valencia, CA), and sequenced by using primer Sqfp (5'-GCCAACGACTACGCACTA GCCA-3') to determine the junction sequence where the Tn meets genomic DNA. The sequence of the junction region of the Tn mutants was searched against the genome sequences of *Salmonella enterica* serovars Enteritidis, Typhi, and Typhimurium, and genes disrupted by Tn insertions were identified (<http://www.sanger.ac.uk/Projects/Microbes/>; <http://www.ncbi.nih.gov>).

A rescue cloning procedure was used as an alternative method to identify the Tn insertion sites in the transposon mutants of *Salmonella enterica* serovar Enteritidis when the three-step PCR procedure failed. Specifically, 10 µg of purified genomic DNA of a mutant was first digested with restriction enzyme SspI and then PvuI and EcoRV concurrently (New England Biolabs, Beverly, MA). Digested DNA was then ligated with T4 DNA ligase (New England Biolabs) and electroporated into *E. coli* pir-116 (Epicentre). Plasmid DNA from the transformants was purified and sequenced with primer Sqfp to obtain the sequence of the junction regions of the Tn and the genome. Genes disrupted by Tn insertions were identified from the sequence of the junction regions as described above for the three-step PCR strategy.

The transposon insertion sites in all mutants were confirmed by designing primers flanking the putative insertion sites, and amplifying genomic DNA of the mutants and wild-type *Salmonella enterica* serovar Enteritidis by PCR. Mutants that yielded a PCR product that was 1.8 kb larger (the size of the transposon) than the PCR product from the wild-type bacteria were considered correct. PCR products from selected mutants were also sequenced to further confirm the site of Tn integration. All insertion sites proved to be correct.

**Determination of the growth curves of the ES mutants and their ability to survive under stress conditions.** The growth of the ES mutants was tested in both LB broth and M9 minimal medium (51). ES mutants were cultured in LB broth at 37°C overnight with shaking. Overnight cultures were diluted 1:100 in LB broth or M9 minimal medium in 96-well plates. Diluted cultures were incubated at 37°C and bacterial concentrations were determined by plating appropriately diluted bacterial cultures on LB agar plates at 0, 2, 4, 8, 12, and 24 h to construct a growth curve. The ability of the ES mutants to survive and grow under general stress conditions was determined by measuring the growth curves of the mutants in LB broth at pH 4.0, LB broth with 1 M NaCl, or LB broth with 5% sodium dodecyl sulfate at 37°C (38).

**Expression of *Salmonella enterica* serovar Enteritidis protein in *Salmonella enterica* serovar Typhimurium.** Primers 5'-ATAGCAACAAGCTTCTACTTC GGTAATGGTGG-3' and 5'-AGACCAGGAAGCTTGATGAGGCCACGC TACACA-3' were used to amplify genomic DNA of *Salmonella enterica* serovar Enteritidis SE2472. The PCR product contained the full coding sequence of SEN4287, the *Salmonella enterica* serovar Enteritidis unique gene disrupted in ES16, and was cloned into the vector pRB3-273C (3) at the HindIII site. The resulting plasmid, pRB3-SEN4287 was transformed into *Salmonella enterica* serovar Typhimurium isolate ST3665 for egg resistance assays.

## RESULTS

***Salmonella enterica* serovar Enteritidis demonstrates better survival in egg albumen than *Salmonella enterica* serovar Typhimurium and *E. coli*.** The association of *Salmonella enterica* serovar Enteritidis with chicken eggs may be due to its enhanced ability to survive in eggs. To explore this possibility, we measured the survival of clinical isolates of *Salmonella enterica* serovars Enteritidis and Typhimurium and animal and laboratory strains of *E. coli* in egg albumen. Overnight cultures of bacteria were mixed with egg albumen and incubated at 37°C for 24 h, and the resistance of bacteria to egg albumen was measured by the survival rate of bacteria after the incubation. A comparison of the survival rates was performed with 15 clinical isolates of *Salmonella enterica* serovar Enteritidis, 10 clinical isolates of *Salmonella enterica* serovar Typhimurium, and 5 *E. coli* strains including the laboratory strains DH5α, HB101, K-12 (MG1665) and 2 animal isolates of *E. coli* (34) (Table 1). After 24 h of incubation in egg albumen, *Salmonella enterica* serovar Enteritidis isolates displayed an average sur-

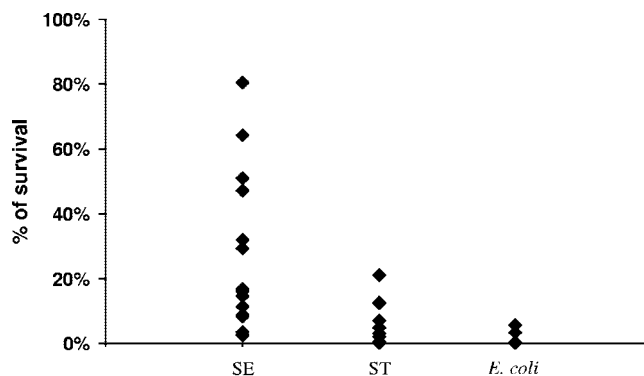


FIG. 1. Survival of clinical isolates of *Salmonella enterica* serovar Enteritidis (SE), *Salmonella enterica* serovar Typhimurium (ST), and animal and laboratory strains of *E. coli* after incubation with egg albumen for 24 h. Each datum point represents an independent bacterial strain listed in Table 1. The percentage of survival represents the ratio of surviving bacteria after 24 h of incubation to the input bacteria. At least three experiments were performed, and results from a representative experiment performed in triplicate are shown.

vival rate of 25.8%, *Salmonella enterica* serovar Typhimurium isolates displayed a rate of 6.5% and *E. coli* isolates displayed a rate of 1.8% (Fig. 1). Thus, *Salmonella enterica* serovar Enteritidis isolates survive better in egg albumen than serovar Typhimurium isolates ( $P = 0.01$ , Student's  $t$  test) and *E. coli* isolates ( $P = 0.002$ , Student's  $t$  test). Although the survival rates of *Salmonella enterica* serovar Typhimurium isolates overlapped with those of *Salmonella enterica* serovar Enteritidis isolates, only *Salmonella enterica* serovar Enteritidis isolates demonstrated high rates of survival (Fig. 1).

**Construction of a transposon mutant library of *Salmonella enterica* serovar Enteritidis.** Since the survival of *Salmonella enterica* serovar Enteritidis in egg albumen is critical for its transmission to human populations and contributes to its epidemiological association with chicken eggs, we decided to use a genetic approach to identify the molecular determinants of *Salmonella enterica* serovar Enteritidis that are necessary for its survival in egg albumen. We used a systematic approach by screening a Tn mutant library to identify genes that play a role in the survival of *Salmonella enterica* serovar Enteritidis in egg albumen.

We used the vector pMOD3, which contains the highly active mosaic ends for transposition, and the origin of replication of R6Kγ for rescue cloning of the adjacent chromosomal DNA to identify genes disrupted by the Tn (Epicentre). Since the Tn coded by pMOD3 was not marked, we first introduced a kanamycin resistance gene ( $Kan^r$ ) into the vector at the EcoRI site. The resulting clone is referred to as pMOD3-Kan (Fig. 2), and it was used to construct the transposon mutant library. We obtained a library with approximately 3,000 individual transposon mutants.

**Screening of a transposon mutant library of *Salmonella enterica* serovar Enteritidis for mutants susceptible to egg albumen.** A total of 2,850 transposon mutants were screened for a decreased ability to survive in egg albumen compared to the wild-type *Salmonella enterica* serovar Enteritidis SE2472 after 24 h of incubation at 37°C. The wild-type SE2472 was included in all screenings as a control and usually demonstrated over

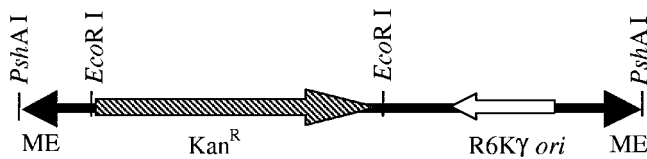


FIG. 2. Transposon used for the construction of the *Salmonella enterica* serovar Enteritidis transposon mutant library. The structure of the transposon encoded by the plasmid pMOD3-Kan is shown. The 19-bp mosaic end sequence for transposition flanks the transposon. R6K $\gamma$  ori was used to rescue the adjacent chromosomal sequence into a plasmid for insertion site identification. The kanamycin resistance cassette (Kan<sup>R</sup>) was cloned into the construct to provide a selection marker. The PstAI sites were used to release the transposon sequence from the vector.

80% survival after 24 h of incubation in egg albumen. Mutants that had less than 10% of survival after the same incubation period were designated potentially susceptible to egg albumen. Sixty mutants were isolated from the screening and were designated as ES mutants (data not shown).

To confirm that the egg albumen susceptibility displayed by the ES mutants obtained from the initial screening was due to Tn insertions instead of unexpected mutations or other changes in the bacteria, we transduced the Tn mutations into fresh *Salmonella enterica* serovar Enteritidis SE2472 by general transduction with phage P22 (39). Phage-free mutants were selected and tested for their survival in egg albumen. Each mutant was tested in at least three independent assays and 36 mutants that were consistently more susceptible to egg albumen than the wild-type parental strain SE2472 were selected (data not shown).

To determine whether the susceptibility to egg albumen displayed by the ES mutants was due to general growth defects that are unrelated to their resistance to egg albumen, we determined the growth curves of the ES mutants in both LB broth and M9 minimal medium. One mutant that displayed defective growth in M9 minimal medium was excluded from further analysis. Thirty-five ES mutants that did not display defects in growth in either LB or M9 medium were further characterized (data not shown).

We identified genes inactivated in the ES mutants by determining the DNA sequence surrounding the Tn insertion sites. The two methods used were a 3-step PCR and a rescue cloning procedure (see Materials and Methods). For each ES mutant, the genomic DNA sequences immediately adjacent to the Tn insertion site were obtained and searched against genome sequences of *Salmonella enterica* serovars Enteritidis, Typhimurium, and Typhi (<http://www.sanger.ac.uk/Projects/Microbes/>; <http://www.ncbi.nih.gov>). In this way, the location of the Tn insertion site in the genome was determined, along with whether a Tn insertion site was located in or near an open reading frame (ORF), as predicted by the annotated genomic sequences. We chose to select ES mutants with a Tn insertion that either disrupted an ORF or was located within 35 bp upstream of an ORF. These types of insertions are expected to disrupt the function of the ORFs involved. Although Tn insertions downstream or further upstream of an ORF may result in phenotypical differences in a mutant by disrupting regulatory sequences or unidentified small RNAs, we decided to focus on those mutants which clearly demonstrated disruptions in ORFs in the present study. The insertion

sites in all mutants were confirmed by PCR with primers adjacent to the identified insertion sites. As a result, 32 ES mutants were selected, and the ORF expected to be disrupted in each mutant are listed in Table 2. Since the *Salmonella enterica* serovar Enteritidis genome has not been fully annotated, genes disrupted by a Tn insertion were named after their *Salmonella enterica* serovar Typhimurium or *Salmonella enterica* serovar Typhi homologs when possible. When a Tn insertion was identified in an un-named gene, the annotation number of the *Salmonella enterica* serovar Enteritidis genome was used (<http://www.sanger.ac.uk/Projects/Microbes/>). One mutant, ES37, had a Tn insertion in a region of the SE2472 genome that does not have good homology to the *Salmonella enterica* serovar Enteritidis genome determined by the Sanger Institute (<http://www.sanger.ac.uk/Projects/Microbes/>). Therefore, the annotation for *Salmonella enterica* serovar Typhimurium was used (<http://www.ncbi.nih.gov>).

**Categorization of the ES mutants according to the functions or putative functions of genes disrupted by Tn insertions.** The ES mutants were categorized according to the functions or putative functions of the ORFs expected to be disrupted in the mutants. Five categories were used: cell wall structure and function, putative cell wall proteins, metabolism, *Salmonella enterica* serovar Enteritidis unique genes, and function unknown (Table 2). Among the ES mutants, 16 of 32 mutants (50.0%) were in the categories of cell wall structure and function or putative cell wall proteins. ES mutants in the cell wall categories by far comprised the largest group of ES mutants (Table 2). Another nine ES mutants had insertions in genes that are involved in metabolism, six of which (ES3, -12, -20, -22, -25, and -52) had a Tn insertion in genes that either encode enzymes or transcription factors involved in amino acid metabolism (ES20, -22, -25, and -52) or show significant homology to proteins involved in amino acid metabolism (ES3 and ES12). Two ES mutants (ES5 and 7) had Tn insertions in genes involved in nucleic acid metabolism. We have also identified two egg-susceptible mutants (ES16 and 47) that had Tn insertions in genes that are unique to *Salmonella enterica* serovar Enteritidis. The seven remaining ES mutants had Tn insertions in genes of unknown function.

The 16 mutants that belonged to the cell wall categories had Tn insertions either in genes known to be involved in cell wall structure and function or in genes that are predicted to encode membrane associated proteins. Among them, 10 mutants (ES1, -2, -10, -11, -15, -21, -30, -46, -53, and -54) had insertions in genes that code for structural proteins, putative structural proteins, or proteins that modulate cell wall properties. In this group, three mutants (ES2, -11, and -46) had Tn insertions in genes involved in the type III secretion (9, 33, 48, 54). Three ES mutants (ES10, -15, and -21) had Tn insertions in genes involved in the transport of amino acids or metal ions. Two ES mutants, ES30 and -53, had Tn insertions in genes involved in fimbrial processing and lipopolysaccharide (LPS) synthesis, respectively (30). One ES mutant, ES54, had a Tn insertion in *yijC*, which has been shown to encode the transcription factor FabR, a regulator of unsaturated fatty acid production and a modulator of the physical properties of the cell membrane of *E. coli* (63). Another mutant, ES1, had a Tn insertion in a gene homologous to a mechanosensitive channel that may regulate

TABLE 2. ES mutants of *S. enterica* serovar Enteritidis SE2472<sup>a</sup>

Category	Mutant	Gene	Function and/or feature
Cell wall structure or function	ES1	SEN3892	Homologous to mechanosensitive ion channel
	ES2	<i>prgH</i>	Component of type III secretion apparatus
	ES10	<i>glnH</i>	Glutamine-binding periplasmic protein precursor
	ES11	<i>prgJ</i>	Invasion protein of type III secretion apparatus
	ES15	<i>proY</i>	Proline-specific permease
	ES21	<i>modF</i>	Putative molybdenum transporter
	ES30	<i>bcfC</i>	Fimbrial usher protein
	ES46	<i>spaP</i>	Membrane protein of type III secretion system
	ES53	<i>waaJ</i>	LPS synthesis
	ES54	<i>yijC</i>	Transcription factor regulating fat production
Putative cell wall proteins	ES17	SEN1188	Putative inner membrane protein
	ES31	<i>yigQ</i>	Putative periplasmic protein or exported protein
	ES33	SEN1861	Putative inner membrane lipoprotein
	ES37	STM3980	Putative outer membrane protein
	ES41	SEN0784	Putative inner membrane protein
	ES50	SEN1204	Putative membrane protein
Metabolism	ES3	<i>ordL</i>	Homologous to DadA involved in phenylalanine metabolism
	ES5	<i>tdk</i>	Thymidine kinase
	ES7	<i>yejD</i>	Ribosomal small subunit pseudouridine synthase
	ES12	<i>ydiB</i>	Putative shikimate 5-dehydrogenase involved in aromatic amino acid synthesis
	ES20	<i>ybdL</i>	Putative aminotransferase involved in phenylalanine metabolism
	ES22	<i>tyrR</i>	Regulator of aromatic amino acid biosynthesis and transport
	ES25	<i>cadA</i>	Lysine decarboxylase
ES52	<i>lysC</i>	Lysine sensitive aspartokinase III	
Unknown function	ES6	SEN2128	Putative cytoplasmic protein
	ES19	SEN2997	Putative ATP-dependent RNA helicase-like protein
	ES27	<i>ygdI</i>	Putative lipoprotein
	ES28	SEN2263	Transcriptional regulator, function unknown
	ES35	<i>ybbN</i>	Putative thioredoxin protein
	ES51	<i>rssC</i>	Putative cytoplasmic protein
SE specific	ES16	SEN4287	Possible restriction endonuclease gene
	ES47	Prot6E gene	Fimbrial biosynthesis

<sup>a</sup> A summary of the characteristics of ES mutants isolated from screening a Tn mutant library for mutants with decreased survival in egg albumen compared to the wild-type *Salmonella enterica* serovar Enteritidis is presented. The gene that was disrupted by the Tn insertion in each mutant is listed. If the Tn insertion was present in a gene that is uncharacterized and unnamed, the annotation of *Salmonella enterica* serovar Enteritidis or *Salmonella enterica* serovar Typhimurium genome is used. Salient features of the ORFs disrupted by the Tn insertion in each mutant are summarized.

cell wall properties in the hyperosmotic environment of egg albumen.

In the second largest category of mutants, the Tn insertions were in genes involved in the metabolism of the bacterium. Of the eight ES mutants in this category, six had Tn insertions in genes involved in amino acid metabolism (ES3, -12, -20, -22, -25, and -52) and two had Tn insertions in genes encoding enzymes that are involved in nucleic acid metabolism (ES5 and -7). Among the ES mutants with disruptions in the metabolism of amino acids, genes disrupted by Tn insertions appear to be involved predominantly in the metabolism of aromatic amino acids and lysine (47; Kegg Pathway Database [http://www.genome.jp/kegg/metabolism.html]). Two ES mutants, ES5 and -7, had insertions in genes encoding thymidine kinase and ribosomal small subunit pseudouridine synthase, respectively.

Two ES mutants (ES16 and -47) had insertions in genes unique to *Salmonella enterica* serovar Enteritidis (Fig. 3). ES16 had a Tn insertion in a gene homologous to a restriction endonuclease. ES47 had a Tn insertion in the Prot6E gene, which is in the *pef* operon that encodes fimbrial proteins in *Salmonella enterica* serovars Enteritidis and Choleraesuis (Fig. 3). According to the genome sequence of *Salmonella enterica*

serovar Enteritidis as determined by the Sanger Institute, the Tn insertion of ES16 was in the genome, while the Tn insertion site of ES47 was in the virulence plasmid (<http://www.sanger.ac.uk/Projects/Microbes/>).

The six ES mutants in the “unknown function” category had Tn insertions in genes with un-identified functions (Table 2). Although some predictions have been made regarding the cellular locations and functions of proteins encoded by these genes, these genes are yet to be characterized.

**Analysis of the egg resistance of a *Salmonella enterica* serovar Typhimurium isolate expressing the gene interrupted in ES16 (SEN4287).** Since the Tn insertions in ES16 and ES47 disrupted genes unique to *Salmonella enterica* serovar Enteritidis, we cloned the SEN4287 and Prot6E genes into the plasmid pRB3-273C and transformed them into *Salmonella enterica* serovar Typhimurium isolate ST3665. The plasmid containing SEN4287, pRB-SEN4287, was transformed into ST3665, and the transformants were assayed for their survival in egg albumen and compared to vector pRB3-273C transformed ST3665, untransformed ST3665, and *Salmonella enterica* serovar Enteritidis isolate SE2472. The plasmid pRB-SEN4287 increased the survival of ST3665 compared to the untransformed or

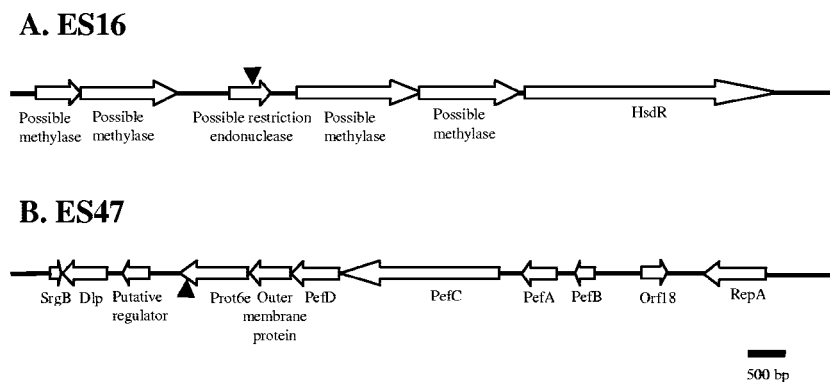


FIG. 3. 3 *Salmonella enterica* serovar Enteritidis unique DNA regions surrounding the transposon insertion sites in ES16 and ES47 mutants. (A) ORFs near the Tn insertion site in ES16; (B) ORFs near the Tn insertion site in ES47. The insertion site in each mutant is marked by an arrowhead.

vector pRB3-273C transformed ST3665 (Student's *t* test,  $P < 0.05$ ) (Fig. 4). However, pRB-SEN4287 did not increase the survival of ST3665 to the level of the *Salmonella enterica* serovar Enteritidis isolate SE2472 (Fig. 4). Plasmid containing the Prot6E gene appeared to be harmful to *Salmonella enterica* serovar Typhimurium. Few colonies were obtained after repeated attempts of transformation, and most of them failed to grow in liquid culture (data not shown).

#### Specificity of egg albumen susceptibility of the ES mutants.

We have described previously that the selected ES mutants had normal growth properties in both complete (LB broth) and minimal medium (M9 minimal medium). To determine whether the susceptibility of the ES mutants to egg albumen is due to their

excessive susceptibility to general stress conditions, we tested the growth and survival of the ES mutants in the presence of low pH (pH 4.0 LB), salt (1 M NaCl in LB), and detergent (5% sodium dodecyl sulfate in LB) (38). ES17 demonstrated less growth in 1 M NaCl compared to the wild-type parental strain, indicating that it has increased susceptibility to salt (data not shown). All other mutants were able to grow to a similar extent as the wild-type SE2472 in all conditions (data not shown), demonstrating that they are not unusually susceptible to stress conditions and that their susceptibility to egg albumen is relatively specific.

## DISCUSSION

**Association of *Salmonella enterica* serovar Enteritidis with chicken eggs.** Over 2,000 serovars of *Salmonella enterica* exist (18); many of which have unique characteristics regarding their host specificity, epidemiology, and pathogenesis (41, 45). The serovar Enteritidis has emerged from a minor serovar in the 1960s to become the second most prevalent *Salmonella* serovar in the United States (2, 6, 24). The most prominent feature of *Salmonella enterica* serovar Enteritidis is its association with intact chicken eggs (2, 5, 6, 24, 32). Here we show that one of the reasons for its association with chicken eggs may be its enhanced ability to survive in chicken egg albumen, an environment that is generally hostile to bacteria (Fig. 1). Compared to the closely related serovar Typhimurium, clinical isolates of Enteritidis demonstrated a statistically significant enhanced survival in egg albumen. The serovar Typhimurium is often transmitted through chicken meat to human populations. It is equally capable of colonizing the reproductive tissues of chickens and forming eggs; however, it rarely causes human infections through intact eggs (32). The differential survival of *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Typhimurium in egg albumen may contribute to the epidemiological differences regarding the food vehicles through which they are transmitted.

The Tn mutant library screening described in the present study isolated two ES mutants, ES16 and ES47, with Tn insertions in regions of the *Salmonella enterica* serovar Enteritidis genome that are absent from the genome of serovar Typhimurium (Table 2 and Fig. 3). These regions may contribute to

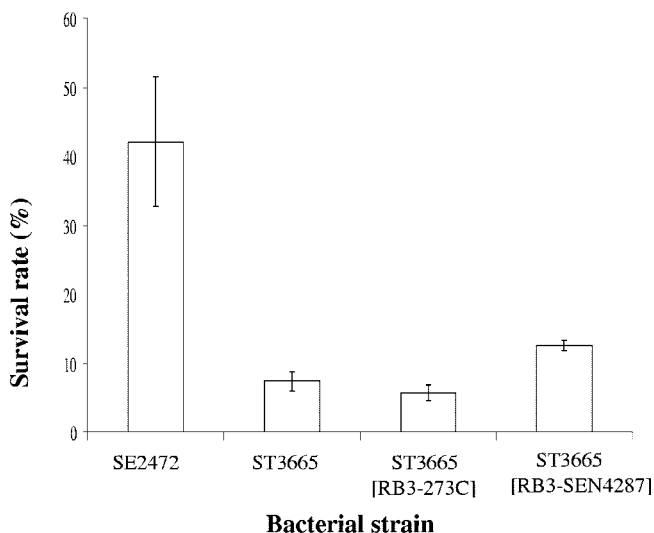


FIG. 4. Survival of *Salmonella enterica* serovar Typhimurium isolate ST3665 derivatives after incubation with egg albumen for 24 h. *Salmonella enterica* serovar Typhimurium ST3665, ST3665 transformed with plasmid vector pRB3-273C (ST3665[RB3-273C]), ST3665 transformed with plasmid containing SEN4287 (ST3665[RB3-SEN4287]), and *Salmonella enterica* serovar Enteritidis SE2472 were incubated with egg albumen at 37°C. The percentage of survival represents the ratio of surviving bacteria after 24 h of incubation to the input bacteria. The results from three independent experiments are shown. Error bars indicate standard deviation.

the unique association of *Salmonella enterica* serovar Enteritidis with chicken eggs. The Tn insertion in ES16 resided in a region of methylases and restriction endonucleases. When the gene interrupted in ES16 (SEN4287) was expressed from a plasmid in the *Salmonella enterica* serovar Typhimurium isolate ST3665, it increased the survival of ST3665. The survival of ST3665 transformed with a plasmid containing SEN4287 was still less than that of the *Salmonella enterica* serovar Enteritidis SE2472, indicating factors other than SEN4287 are also important for survival in egg albumen (Fig. 4). The mechanism of how SEN4287 affects the survival of *Salmonella enterica* serovar Enteritidis in egg albumen is yet to be determined. ES47 had a Tn insertion in the Prot6E, adjacent to the *pef* operon. Although the *pef* operon is also present in the virulence plasmid of *Salmonella enterica* serovar Typhimurium, Prot6E is absent from the genome and the plasmid of *Salmonella enterica* serovar Typhimurium. Prot6E may provide unique surface fimbriae to *Salmonella enterica* serovar Enteritidis and alter its interaction with egg albumen components. This is consistent with our findings that alteration of the surface properties of *Salmonella enterica* serovar Enteritidis may render the bacterium more susceptible to egg albumen (to be discussed below).

Closely related serovars of *Salmonella* are reported to have a 10 to 12% difference between their genomes. Statistically, three to four mutants among the 32 ES mutants are expected to have a Tn insertion in regions of the serovar Enteritidis genome that are absent in the serovar Typhimurium genome. In addition to ES16 and ES47 that had Tn insertion in regions of genome that are absent in the serovar Typhimurium, a third mutant also had a Tn insertion in an Enteritidis unique region that encodes a small ORF of 42 amino acids with no significant similarities to any protein in the database (data not shown). The mutant had growth defects in M9 minimal medium and was excluded from this report. Therefore, we did not detect a disproportionately large number of ES mutants that had Tn insertions in genes unique to *Salmonella enterica* serovar Enteritidis.

**Interaction of *Salmonella enterica* serovar Enteritidis with egg albumen.** The chemical nature of the antimicrobial activities of egg albumen and how egg albumen controls bacteria are not well understood. In the present study, we used the transposon mutagenesis approach to identify *Salmonella enterica* serovar Enteritidis genes that are necessary for it to persist in egg albumen. None of the genes in this report appeared to be necessary for the growth or survival of *Salmonella enterica* serovar Enteritidis in laboratory media or general stress conditions such as low pH, detergent and salt, suggesting that their functions in survival in egg albumen are relatively specific. Those genes should provide insight into how *Salmonella enterica* serovar Enteritidis interacts with egg albumen and survives.

Egg albumen was previously reported to restrict the growth of *Salmonella enterica* serovar Enteritidis (1, 8, 22), and we have demonstrated that egg albumen is bactericidal toward *Salmonella* (37). Egg albumen was reported to contain multiple antimicrobial components, such as high concentrations of ovotransferrin and lysozyme (58). The principal antimicrobial component of the egg albumen is believed to be ovotransferrin that chelates iron that is necessary for bacteria to proliferate (1, 7, 58). More recently, both ovotransferrin and lysozyme

were reported to form pores in gram-negative bacteria through cationic peptides (28, 29, 46). Moreover, we have discovered that egg albumen may also damage bacterial DNA. A DNA repair enzyme, exonuclease III, and a putative DNA repair protein, YafD, are necessary for *Salmonella enterica* serovar Enteritidis to survive in egg albumen (37). We propose that egg albumen controls bacteria by (i) inhibiting bacterial growth through iron chelation by ovotransferrin and (ii) killing bacteria through the direct interaction of egg albumen components with the bacterial cell wall and possible access to the cytoplasm through some of those interactions. This hypothesis is supported by our finding that a large number of egg-susceptible mutants isolated from our transposon library screening have Tn insertions in genes involved in the structure and function of the cell wall, which suggests that the direct interaction of bacteria with egg albumen components determine *Salmonella enterica* serovar Enteritidis' ability to survive in egg albumen.

**Role of the cell wall in the survival of *Salmonella enterica* serovar Enteritidis in egg albumen.** Of the 32 ES mutants we have isolated, 25 (78.1%) had a Tn insertion in genes involved in either cell wall structure/function or amino acid metabolism. In addition, these two systems are related to each other. For example, ES10 and ES15 had insertions in cell wall proteins that function in the transport of glutamine and proline, respectively. ES25 and ES52, which had Tn insertions in genes involved in lysine and cadaverine metabolism, were likely to have had a cadaverine deficiency leading to destabilized LPS of *Salmonella* (to be discussed below). Therefore, cell wall structural and functional integrity and amino acid metabolism are essential for *Salmonella enterica* serovar Enteritidis to survive the stress of exposure to egg albumen.

The cell wall of gram-negative bacteria is composed of an outer membrane, a peptidoglycan layer, and a cytoplasmic membrane. LPS is on the surface of the gram-negative bacteria, making it the first barrier of the bacterial cell wall. It is believed that positively charged cationic peptides can interact with the negatively charged LPS leading to the insertion of the peptides into the membranes (19, 25, 42). Such cationic peptides are present in egg albumen. Both ovotransferrin and lysozyme have been demonstrated to have cationic activity and can form pores in the membranes of gram-negative bacteria (28, 29, 46). Any change in the charge or structure of LPS in *Salmonella enterica* serovar Enteritidis could alter its interaction with ovotransferrin, lysozyme, or other cationic peptides and cause *Salmonella enterica* serovar Enteritidis to become more susceptible to egg albumen. This is consistent with our observation that ES53, which had a Tn insertion in *waaJ*, a gene involved in LPS biosynthesis, is susceptible to egg albumen. We have also isolated mutants ES25 and ES52, which were expected to be defective in lysine and cadaverine metabolism, respectively. ES25 was defective in secreting cadaverine (R. Clavijo and S. Lu, unpublished observations) and ES52 was expected to be defective in lysine biosynthesis which is necessary for cadaverine production. Cadaverine is an outer membrane constituent of *E. coli* and *Salmonella* and has a stabilizing effect on the LPS of gram-negative bacteria (35, 60). Without the stabilizing effect of the positively charged cadaverine, the mutants ES25 and ES52 might have LPS that is less stable than in the wild-type bacteria and are therefore ren-

dered more vulnerable to the activities of the cationic peptides in egg albumen.

In addition to changes in LPS, any mutation that changes the surface properties (e.g., charge, integrity) of *Salmonella enterica* serovar Enteritidis may potentially render it more susceptible to the antimicrobial activities of ovotransferrin, lysozyme and other yet unidentified antimicrobial components of egg albumen. This may be the reason why ES30 and ES54 were susceptible to egg albumen. ES30 had a Tn insertion in *bcfC* which encodes a fimbrial usher protein, and ES54 had Tn insertion in *yijC*, which encodes FabR, a transcription factor that regulates lipid metabolism and modulates membrane properties (63). We have also unexpectedly isolated three ES mutants (ES2, ES11, and ES46) that had Tn insertions in genes encoding the structural components of the type III secretion system. The type III secretion system of *Salmonella* is believed to be activated by contact with host cells, leading to the secretion of effector molecules to mediate the invasion and trafficking of *Salmonella* and the immunomodulation of host cells (9, 20, 33). The environment of egg albumen is significantly different from that of the host cell membrane, and if the type III secretion system is activated by egg albumen, the quantity of the effector molecules secreted is expected to be too low to affect the bactericidal activity of egg albumen. The proteins encoded by the *prg* operon of the type III secretion system are expressed in cultured *Salmonella* not exposed to host cells (33, 54); therefore, it is possible that the type III secretion system has as-yet-unidentified functions in the absence of host cells. Recently, Rietsch et al. reported that the metabolic state of histidine of *Pseudomonas aeruginosa* influences expression of the type III regulon (49). This suggests that the type III secretion system and metabolism of amino acids are connected.

Egg albumen has a high concentration of proteins (ca. 250 mg/ml; S. Lu, unpublished results) that represents a hyperosmotic environment for bacteria. Two of the ES mutants we isolated (ES10 and ES15) had Tn insertions in genes involved in glutamine and proline transport, respectively. Proline transport systems facilitate bacteria's survival in hyperosmotic conditions because proline is an osmoprotectant that bacteria accumulate to high concentrations to counter the extracellular hyperosmolarity (4, 53, 57, 61). *Salmonella* has other proline transport systems, including a high-affinity proline transport system encoded by the *putP* gene, and two glycine betaine transport systems with a lower affinity for proline encoded by the *proP* and *proU* genes (17, 62). ES15 shows normal growth in LB broth with 1 M NaCl; however, the impact of the ProY inactivation on *Salmonella enterica* serovar Enteritidis' survival in other hyperosmotic conditions is yet to be determined. Glutamine was shown to be an osmoprotectant in a soil enterobacterium *Erwinia chrysanthemi* that is closely related to *Escherichia coli* (23). *E. chrysanthemi* accumulates glutamine under hyperosmotic conditions (23); it remains to be seen whether the same is true for *Salmonella*.

**Role of amino acid metabolism in the survival of *Salmonella enterica* serovar Enteritidis in egg albumen.** Amino acid metabolism mutants constituted a large percentage of the ES mutants (6 of 32, or 18.8%). Interestingly, all ORFs inactivated in these mutants appeared to be involved in the metabolism of either lysine (ES25 and ES52) or aromatic amino acids, especially phenylalanine (ES3, ES12, ES20, and ES22). Lysine and

cadaverine metabolism may be involved in maintaining the LPS integrity as discussed previously. The metabolism of aromatic amino acids is important for many aspects of pathogenic bacteria. Inactivation of aromatic pathways is used to attenuate *Salmonella* for vaccines for both human and farm animals (11, 36, 50, 55). The shikimate pathway that is responsible for aromatic amino acid biosynthesis is also necessary for the synthesis of a variety of important biomolecules including enterobactin, folates, benzoids, and naphthoids (14). It would be interesting to determine whether the cellular concentrations of aromatic amino acids and other molecules affected by the shikimate pathway in the ES mutants are altered compared to the wild-type SE2472 and whether other genes in aromatic amino acid metabolism pathways are also involved in the survival of *Salmonella enterica* serovar Enteritidis in egg albumen.

#### **Serovar specificity of *Salmonella enterica* serovar Enteritidis.**

In our studies, we have found that clinical isolates of *Salmonella enterica* serovar Enteritidis are generally more resistant to egg albumen than those of *Salmonella enterica* serovar Typhimurium (Fig. 1). This may be one of the reasons why *Salmonella enterica* serovar Enteritidis transmits to humans through intact chicken eggs, whereas *Salmonella enterica* serovar Typhimurium only transmits through cracked eggs occasionally. This indicates that *Salmonella enterica* serovar Enteritidis may possess characteristics that enhance its survival in egg albumen. Three possibilities exist for the increased resistance of *Salmonella enterica* serovar Enteritidis to egg albumen and its unique association with chicken eggs: (i) *Salmonella enterica* serovar Enteritidis has a unique set of "egg-resistant genes" that are absent in *Salmonella enterica* serovar Typhimurium; (ii) sequence diversity in genes present in both *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Typhimurium result in significant differences in their functions in egg albumen resistance; and (iii) differential regulation of genes shared between *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis lead to the increased resistance of *Salmonella enterica* serovar Enteritidis. We have isolated only two *Salmonella enterica* serovar Enteritidis unique genes among 32 egg-susceptible mutants, a number that is expected if the genome of the serovar Enteritidis differs from that of the serovar Typhimurium by 10 to 12% (16). This indicates that *Salmonella enterica* serovar Enteritidis is unlikely to possess an extensive collection of unique genes that increase its survival in egg albumen. Therefore, the first possibility is unlikely. We had also compared the sequences of the ORFs disrupted in the ES mutants to their homologs in *Salmonella enterica* serovar Typhimurium and found that the homology of all of the ORFs was between 98 and 100% (data not shown). Although it cannot be ruled out that sequence heterogeneities in key amino acids result in different functions of the proteins encoded by these ORFs, the high homology between the *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Typhimurium proteins argues against the second possibility. Therefore, the most likely explanation for the difference in survival of *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Typhimurium in egg albumen may be their differential regulation of genes in response to egg albumen. A profiling analysis of mRNA and protein expression of multiple strains of *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Typhi-

murium exposed to egg albumen will allow us to determine whether genes necessary for *Salmonella* to survive in egg albumen are differentially regulated in *Salmonella enterica* serovars Enteritidis and Typhimurium.

In conclusion, we have identified here genes that play significant roles in the survival of *Salmonella enterica* serovar Enteritidis in egg albumen. These genes might represent significant factors that had led *Salmonella enterica* serovar Enteritidis to become a major food-borne pathogen over the past few decades. Identification of genes necessary for the egg resistance of *Salmonella enterica* serovar Enteritidis has improved our understanding of the molecular basis behind *Salmonella enterica* serovar Enteritidis' ability to survive in chicken eggs, as well as the possible antimicrobial activities of egg albumen that have not yet been fully characterized. Genes that are necessary for *Salmonella enterica* Serovar Enteritidis to persist in egg albumen may be useful for generating safer live, attenuated vaccines for *Salmonella enterica* serovar Enteritidis. Mutations in these genes can be introduced into *Salmonella enterica* serovar Enteritidis vaccine strains to reduce the possibility of egg contamination by the vaccine strains and therefore make them safer for consumers.

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#### REFERENCES

- Baron, F., M. Gautier, and G. Brule. 1997. Factors involved in the inhibition of growth of *Salmonella enteritidis* in liquid egg white. *J. Food Prot.* **60**:1318–1323.
- Baumler, A. J., B. M. Hargis, and R. M. Tsois. 2000. Tracing the origins of *Salmonella* outbreaks. *Science* **287**:50–52.
- Berggren, R. E., A. Wunderlich, E. Ziegler, M. Schleicher, R. C. Duke, D. Looney, and F. C. Fang. 1995. HIV gp120-specific cell-mediated immune responses in mice after oral immunization with recombinant *Salmonella*. *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.* **10**:489–495.
- Cayley, S., B. A. Lewis, and M. T. Record, Jr. 1992. Origins of the osmoprotective properties of betaine and proline in *Escherichia coli* K-12. *J. Bacteriol.* **174**:1586–1595.
- Centers for Disease Control and Prevention. 2003. Outbreaks of *Salmonella* serotype enteritidis infection associated with eating shell eggs—United States, 1999–2001. *JAMA* **289**:540–541.
- Centers for Disease Control and Prevention. 2003. *Salmonella*—annual summary 2002. Centers for Disease Control and Prevention, Atlanta, Ga.
- Chart, H., and B. Rowe. 1993. Iron restriction and the growth of *Salmonella enteritidis*. *Epidemiol. Infect.* **110**:41–47.
- Cogan, T. A., F. Jorgensen, H. M. Lappin-Scott, C. E. Benson, M. J. Woodward, and T. J. Humphrey. 2004. Flagella and curli fimbriae are important for the growth of *Salmonella enterica* serovars in hen eggs. *Microbiology* **150**:1063–1071.
- Collazo, C. M., and J. E. Galan. 1996. Requirement for exported proteins in secretion through the invasion-associated type III system of *Salmonella typhimurium*. *Infect. Immun.* **64**:3524–3531.
- Crump, J. A., S. P. Luby, and E. D. Mintz. 2004. The global burden of typhoid fever. *Bull. W. H. O.* **82**:346–353.
- Curtiss, R., III, S. M. Kelly, and J. O. Hassan. 1993. Live oral avirulent *Salmonella* vaccines. *Vet. Microbiol.* **37**:397–405.
- Datsenko, K. A., and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* **97**:6640–6645.
- Deng, W., S. R. Liou, G. Plunkett III, G. F. Mayhew, D. J. Rose, V. Burland, V. Kodoyianni, D. C. Schwartz, and F. R. Blattner. 2003. Comparative genomics of *Salmonella enterica* serovar Typhi strains Ty2 and CT18. *J. Bacteriol.* **185**:2330–2337.
- Dosselaere, F., and J. Vanderleyden. 2001. A metabolic node in action: chorismate-utilizing enzymes in microorganisms. *Crit. Rev. Microbiol.* **27**:75–131.
- Ducey, T. F., and D. W. Dyer. 2002. Rapid identification of EZ::TN™ transposon insertion sites in the genome of *Neisseria gonorrhoeae*. *EPICENTRE Forum* **9**:6–7.
- Edwards, R. A., G. J. Olsen, and S. R. Maloy. 2002. Comparative genomics of closely related salmonellae. *Trends Microbiol.* **10**:94–99.
- Ekena, K., M. K. Liao, and S. Maloy. 1990. Activation of a new proline transport system in *Salmonella typhimurium*. *J. Bacteriol.* **172**:2940–2945.
- Euzely, J. P. 1999. Revised *Salmonella* nomenclature: designation of *Salmonella enterica*: request for an opinion. *Int. J. Syst. Bacteriol.* **49**(Pt. 2):927–930.
- Farnaud, S., C. Spiller, L. C. Moriarty, A. Patel, V. Gant, E. W. Odell, and R. W. Evans. 2004. Interactions of lactoferricin-derived peptides with LPS and antimicrobial activity. *FEMS Microbiol. Lett.* **233**:193–199.
- Galan, J. E. 2001. *Salmonella* interactions with host cells: type III secretion at work. *Annu. Rev. Cell Dev. Biol.* **17**:53–86.
- Gast, R. K., and P. S. Holt. 2000. Influence of the level and location of contamination on the multiplication of *Salmonella enteritidis* at different storage temperatures in experimentally inoculated eggs. *Poult. Sci.* **79**:559–563.
- Gast, R. K., and P. S. Holt. 2001. Multiplication in egg yolk and survival in egg albumen of *Salmonella enterica* serotype Enteritidis strains of phage types 4, 8, 13a, and 14b. *J. Food Prot.* **64**:865–868.
- Goude, R., S. Renaud, S. Bonnassie, T. Bernard, and C. Blanco. 2004. Glutamine, glutamate, and alpha-glucosylglycerate are the major osmotic solutes accumulated by *Erwinia chrysanthemi* strain 3937. *Appl. Environ. Microbiol.* **70**:6535–6541.
- Guard-Petter, J. 2001. The chicken, the egg and *Salmonella enteritidis*. *Environ. Microbiol.* **3**:421–430.
- Hancock, R. E., and R. Lehrer. 1998. Cationic peptides: a new source of antibiotics. *Trends Biotechnol.* **16**:82–88.
- Henzler, D. J., E. Ebel, J. Sanders, D. Kradel, and J. Mason. 1994. *Salmonella enteritidis* in eggs from commercial chicken layer flocks implicated in human outbreaks. *Avian Dis.* **38**:37–43.
- Humphrey, T. J., A. Whitehead, A. H. Gawler, A. Henley, and B. Rowe. 1991. Numbers of *Salmonella enteritidis* in the contents of naturally contaminated hens' eggs. *Epidemiol. Infect.* **106**:489–496.
- Ibrahim, H. R., T. Matsuzaki, and T. Aoki. 2001. Genetic evidence that antibacterial activity of lysozyme is independent of its catalytic function. *FEBS Lett.* **506**:27–32.
- Ibrahim, H. R., U. Thomas, and A. Pellegrini. 2001. A helix-loop-helix peptide at the upper lip of the active site cleft of lysozyme confers potent antimicrobial activity with membrane permeabilization action. *J. Biol. Chem.* **276**:43767–43774.
- Kaniuk, N. A., E. Vinogradov, J. Li, M. A. Monteiro, and C. Whitfield. 2004. Chromosomal and plasmid-encoded enzymes are required for assembly of the R3-type core oligosaccharide in the lipopolysaccharide of *Escherichia coli* O157:H7. *J. Biol. Chem.*
- Keller, L., C. Benson, K. Krotec, and R. Eckroade. 1995. *Salmonella enteritidis* colonization of the reproductive tract and forming and freshly laid eggs of chickens. *Infect. Immun.* **63**:2443–2449.
- Keller, L. H., D. M. Schifferli, C. E. Benson, S. Aslam, and R. J. Eckroade. 1997. Invasion of chicken reproductive tissues and forming eggs is not unique to *Salmonella enteritidis*. *Avian Dis.* **41**:535–539.
- Kimbrough, T. G., and S. I. Miller. 2000. Contribution of *Salmonella typhimurium* type III secretion components to needle complex formation. *Proc. Natl. Acad. Sci. USA* **97**:11008–11013.
- Kimura, R., R. E. Mandrell, J. C. Galland, D. Hyatt, and L. W. Riley. 2000. Restriction-site-specific PCR as a rapid test to detect enterohemorrhagic *Escherichia coli* O157:H7 strains in environmental samples. *Appl. Environ. Microbiol.* **66**:2513–2519.
- Koski, P., and M. Vaara. 1991. Polyamines as constituents of the outer membranes of *Escherichia coli* and *Salmonella typhimurium*. *J. Bacteriol.* **173**:3695–3699.
- Lockman, H. A., and R. Curtiss III. 1990. Occurrence of secondary attenuating mutations in avirulent *Salmonella typhimurium* vaccine strains. *J. Infect. Dis.* **162**:1397–1400.
- Lu, S., P. B. Killoran, and L. W. Riley. 2003. Association of *Salmonella enterica* serovar Enteritidis *yaqD* with resistance to chicken egg albumen. *Infect. Immun.* **71**:6734–6741.
- Lu, S., A. R. Manges, Y. Xu, F. C. Fang, and L. W. Riley. 1999. Analysis of virulence of clinical isolates of *Salmonella enteritidis* in vivo and in vitro. *Infect. Immun.* **67**:5651–5657.
- Maloy, S. R., V. J. Stewart, and R. K. Taylor. 1996. Genetic analysis of pathogenic bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- McClelland, M., K. E. Sanderson, S. W. Clifton, P. Latreille, S. Porwollik, A. Sabo, R. Meyer, T. Bieri, P. Ozersky, M. McLellan, C. R. Harkins, C. Wang, C. Nguyen, A. Berghoff, G. Elliott, S. Kohlberg, C. Strong, F. Du, J. Carter, C. Kremizki, D. Layman, S. Leonard, H. Sun, L. Fulton, W. Nash, T. Miner, P. Minx, K. Delehaunty, C. Fronick, V. Magrini, M. Nhan, V. Warren, L. Florea, J. Spieth, and R. K. Wilson. 2004. Comparison of genome degradation in Paratyphi A and Typhi, human-restricted serovars of *Salmonella enterica* that cause typhoid. *Nat. Genet.* **36**:1268–1274.

41. McClelland, M., K. E. Sanderson, J. Spieth, S. W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, F. Du, S. Hou, D. Layman, S. Leonard, C. Nguyen, K. Scott, A. Holmes, N. Grewal, E. Mulvaney, E. Ryan, H. Sun, L. Florea, W. Miller, T. Stoneking, M. Nhan, R. Waterston, and R. K. Wilson. 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. *Nature* **413**:852–856.
42. McCoy, A. J., H. Liu, T. J. Falla, and J. S. Gunn. 2001. Identification of *Proteus mirabilis* mutants with increased sensitivity to antimicrobial peptides. *Antimicrob. Agents Chemother.* **45**:2030–2037.
43. Mishu, B., J. Kochler, L. A. Lee, D. Rodrigue, F. H. Brenner, P. Blake, and R. V. Tauxe. 1994. Outbreaks of *Salmonella enteritidis* infections in the United States, 1985–1991. *J. Infect. Dis.* **169**:547–552.
44. Olsen, S. J., L. C. MacKinnon, J. S. Goulding, N. H. Bean, and L. Slutsker. 2000. Surveillance for foodborne-disease outbreaks—United States, 1993–1997. *Morb. Mortal. Wkly. Rep. CDC Surv. Summ.* **49**:1–62.
45. Parkhill, J., G. Dougan, K. D. James, N. R. Thomson, D. Pickard, J. Wain, C. Churcher, K. L. Mungall, S. D. Bentley, M. T. Holden, M. Sebaihia, S. Baker, D. Basham, K. Brooks, T. Chillingworth, P. Connor, A. Cronin, P. Davis, R. M. Davies, L. Dowd, N. White, J. Farrar, T. Feltwell, N. Hamlin, A. Haque, T. T. Hien, S. Holroyd, K. Jagels, A. Krogh, T. S. Larsen, S. Leather, S. Moule, P. O’Gaora, C. Parry, M. Quail, K. Rutherford, M. Simmonds, J. Skelton, K. Stevens, S. Whitehead, and B. G. Barrell. 2001. Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. *Nature* **413**:848–852.
46. Pellegrini, A., U. Thomas, P. Wild, E. Schraner, and R. von Fellenberg. 2000. Effect of lysozyme or modified lysozyme fragments on DNA and RNA synthesis and membrane permeability of *Escherichia coli*. *Microbiol. Res.* **155**:69–77.
47. Pittard, J., H. Camakaris, and J. Yang. 2005. The TyrR regulon. *Mol. Microbiol.* **55**:16–26.
48. Pucciarelli, M. G., and F. Garcia-del Portillo. 2003. Protein-peptidoglycan interactions modulate the assembly of the needle complex in the *Salmonella* invasion-associated type III secretion system. *Mol. Microbiol.* **48**:573–585.
49. Rietsch, A., M. C. Wolfgang, and J. J. Mekalanos. 2004. Effect of metabolic imbalance on expression of type III secretion genes in *Pseudomonas aeruginosa*. *Infect. Immun.* **72**:1383–1390.
50. Robertsson, J. A., A. A. Lindberg, S. Hoiseth, and B. A. Stocker. 1983. *Salmonella typhimurium* infection in calves: protection and survival of virulent challenge bacteria after immunization with live or inactivated vaccines. *Infect. Immun.* **41**:742–750.
51. Sambrook, J., and D. W. Russell. 2001. *Molecular cloning: a laboratory manual*, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
52. Shivaprasad, H. L., J. F. Timoney, S. Morales, B. Lucio, and R. C. Baker. 1990. Pathogenesis of *Salmonella enteritidis* infection in laying chickens. I. Studies on egg transmission, clinical signs, fecal shedding, and serologic responses. *Avian Dis.* **34**:548–557.
53. Spiegelhalter, F., and E. Bremer. 1998. Osmoregulation of the *opuE* proline transport gene from *Bacillus subtilis*: contributions of the sigma A- and sigma B-dependent stress-responsive promoters. *Mol. Microbiol.* **29**:285–296.
54. Sukhan, A., T. Kubori, and J. E. Galan. 2003. Synthesis and localization of the *Salmonella* SPI-1 type III secretion needle complex proteins PrgI and PrgJ. *J. Bacteriol.* **185**:3480–3483.
55. Tacket, C. O., D. M. Hone, R. Curtiss III, S. M. Kelly, G. Losonsky, L. Guers, A. M. Harris, R. Edelman, and M. M. Levine. 1992. Comparison of the safety and immunogenicity of  $\Delta$ aroC  $\Delta$ aroD and  $\Delta$ cya  $\Delta$ crp *Salmonella typhi* strains in adult volunteers. *Infect. Immun.* **60**:536–541.
56. Todd, E. C. 1997. Epidemiology of food-borne diseases: a worldwide review. *World Health Stat. Q.* **50**:30–50.
57. Townsend, D. E., and B. J. Wilkinson. 1992. Proline transport in *Staphylococcus aureus*: a high-affinity system and a low-affinity system involved in osmoregulation. *J. Bacteriol.* **174**:2702–2710.
58. Tranter, H. S., and R. G. Board. 1982. The antimicrobial defense of avian eggs: biological perspective and chemical basis. *J. Appl. Biochem.* **4**:295–338.
59. U.S. Department of Health Services. 2002. *An atlas of Salmonella in the United States*. U.S. Department of Health Services, Washington, D.C.
60. Vaara, M. 1992. Agents that increase the permeability of the outer membrane. *Microbiol. Rev.* **56**:395–411.
61. Whatmore, A. M., J. A. Chudek, and R. H. Reed. 1990. The effects of osmotic upshock on the intracellular solute pools of *Bacillus subtilis*. *J. Gen. Microbiol.* **136**:2527–2535.
62. Wood, J. M. 1988. Proline porters effect the utilization of proline as nutrient or osmoprotectant for bacteria. *J. Membr. Biol.* **106**:183–202.
63. Zhang, Y. M., H. Marrakchi, and C. O. Rock. 2002. The FabR (YijC) transcription factor regulates unsaturated fatty acid biosynthesis in *Escherichia coli*. *J. Biol. Chem.* **277**:15558–15565.