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rpoS-Regulated Core Genes Involved in the Competitive Fitness of *Salmonella enterica* Serovar Kentucky in the Intestines of Chickens

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***Salmonella enterica* serovar Kentucky has become the most frequently isolated serovar from poultry in the United States over the past decade. Despite its prevalence in poultry, it causes few human illnesses in the United States. The dominance of *S.* Kentucky in poultry does not appear to be due to single introduction of a clonal strain, and its reduced virulence appears to correlate with the absence of virulence genes *grvA*, *sseI*, *sopE*, and *sodC1*. *S.* Kentucky's prevalence in poultry is possibly attributable to its metabolic adaptation to the chicken cecum. While there were no difference in the growth rate of *S.* Kentucky and *S.* Typhimurium grown microaerophilically in cecal contents, *S.* Kentucky persisted longer when chickens were coinfected with *S.* Typhimurium. The *in vivo* advantage that *S.* Kentucky has over *S.* Typhimurium appears to be due to differential regulation of core *Salmonella* genes via the stationary-phase sigma factor *rpoS*. Microarray analysis of *Salmonella* grown in cecal contents *in vitro* identified several metabolic genes and motility and adherence genes that are differentially activated in *S.* Kentucky. The contributions of four of these operons (*mgl*, *prp*, *nar*, and *csg*) to *Salmonella* colonization in chickens were assessed. Deletion of *mgl* and *csg* reduced *S.* Kentucky persistence in competition studies in chickens infected with wild-type or mutant strains. Subtle mutations affecting differential regulation of core *Salmonella* genes appear to be important in *Salmonella*'s adaptation to its animal host and especially for *S.* Kentucky's emergence as the dominant serovar in poultry.**

According to the Centers for Disease Control and Prevention, an estimated 1 million cases of human disease due to salmonellosis occur annually in the United States (1). *Salmonella* outbreaks are often associated with the consumption of contaminated meat and eggs (2). While more than 2,000 *Salmonella* serovars have been identified, 20 *Salmonella* serovars account for 73% of salmonellosis cases in the United States (3). *Salmonella enterica* Enteritidis and *S. enterica* serovar Typhimurium are the top two serovars associated with outbreaks in the United States (3) and are responsible for 50% of human infections worldwide (4). The serovar *S.* Kentucky is rarely associated with human illnesses in the United States (0.18% of cases) (3); however, it is the most prevalent serovar in poultry in the United States (5, 6).

The emergence of *S.* Kentucky as the dominant serovar in poultry is similar to the emergence of *S.* Enteritidis in the 1980s (7), except that *S.* Enteritidis quickly became the leading cause of human salmonellosis in many countries (8), including the United States (9). There has been extensive research on *S.* Enteritidis, focused especially on identifying factors that contribute to colonization and egg transmission (10). While *S. enterica* subspecies I serovars share many metabolic and virulence pathways, forming a *Salmonella* core genome (11, 12), there are differences with regard to the distribution of mobile genetic elements (prophages and plasmids) (13, 14), metabolic genes (15), fimbrial operons (16, 17), and certain pathogenicity islands (18) that have shaped their behavior and adaptation to their animal hosts. For example, there are distinct differences in the distributions of specific genes and operons between *S.* Enteritidis and *S.* Typhimurium that may partly explain the differences in egg transmission (19). A comparison of the *S.* Kentucky genome to the reference *S.* Typhimurium strain LT2 reveals similar genomic differences. *Salmonella* Kentucky contains five genomic islands (GenBank accession number ABAK02000001.1), ranging in size from 4 to 18 kb, and two conjugative plasmids (20, 21). One of the genomic islands was predicted to encode several transporters, a glucokinase, a sugar phosphate isomerase, and a carbohydrate-selective porin, suggesting that *S.* Kentucky possesses a metabolic advantage. In addition to resistance to antibiotics, one plasmid was identified as a ColV plasmid that, in addition to carrying genes for colicins, also harbored iron-scavenging genes and the HlyF hemolysin initially characterized in avian pathogenic *Escherichia coli* (APEC) (20, 21). However, there have also been subtler genetic changes, including mutations in core *Salmonella* genes, that have altered virulence and colonization of some *Salmonella* serovars (22–25). *S.* Kentucky exhibits differences in acid tolerance compared to other *Salmonella* serovars and strains (26). Such phenotypic differences exhibited by *Salmonella* strains have been explained by mutations that alter expression of key genes central to a metabolic pathway,

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virulence, or survival mechanisms (27, 28). While there are significant genomic differences between *Salmonella* Kentucky and *S.* Typhimurium that might explain *S.* Kentucky's adaptations, we sought to characterize changes in *Salmonella*'s core metabolism that would explain *S.* Kentucky's emergence as a poultry-adapted serovar.

MATERIALS AND METHODS

Bacterial strains. A total of 216 *S.* Kentucky isolates were examined, consisting of 210 isolates from chicken sources; 4 isolates were from humans and were obtained from the Centers for Disease Control and Prevention (CDC), and 2 isolates were from bovine sources. An additional 168 *Salmonella* isolates were included for comparison and represented 14 different *Salmonella* serovars: *S.* Agona ($n = 5$), *S.* Enteritidis ($n = 9$), *S.* Hadar ($n = 1$), *S.* Heidelberg ($n = 80$), *S.* Infantis ($n = 3$), *S.* Litchfield ($n = 14$), *S.* Mbandaka ($n = 3$), *S.* Montevideo ($n = 11$), *S.* Ohio ($n = 1$), *S.* Oranienberg ($n = 7$), *S.* Schwarzengrund ($n = 3$), *S.* Senftenberg ($n = 6$), *S.* Typhimurium ($n = 15$), and *S.* Worthington ($n = 10$). These *Salmonella* isolates were collected from commercial chickens from multiple companies located in the southeastern United States (6, 29).

Growth rate comparisons. The growth kinetics of *S.* Kentucky isolates ($n = 15$), representative of the different pulsed-field gel electrophoresis (PFGE) types identified in this study, and *S.* Typhimurium strains ($n = 16$) were determined in minimal M9 medium containing 0.1% cecal content as a carbon and energy source. The cecal contents were obtained from 10 broiler chickens (42 days of age) fed diets without growth-promoting antibiotics. After euthanasia, the ceca were collected and opened longitudinally, and the cecal content was collected and sterilized by autoclaving for 15 min. Initial inocula for *Salmonella* cultures were prepared by growing the bacteria in Luria-Bertani (LB) broth at 37°C, aerobically, overnight. One microliter of culture was used to inoculate 1 ml of minimal M9 medium containing cecal contents, in a Bactometer module (bioMérieux, Hazelwood, MI). Uninoculated medium served as a negative control. Strains were grown in quadruplicate at 37°C under microaerophilic conditions (6% CO_2, 6% O_2, 85%N_2, 3% H_2) using a Bactometer model 32 (bioMérieux). Changes in the conductance, related to bacterial growth, were monitored continuously at 6-min intervals for 100 h. When collecting impedance data, it is common to encounter an initial "shut-eye" period, during which impedance values are not reliable. In this data set, impedance values from the first 10 to 20 h were considered part of this "shut-eye" period and were therefore omitted from analysis.

The percent change in impedance was calculated as follows: % change $= (x - x_1)/x_1$, where x is the impedance at a given time point and x_1 is the impedance at the initial time point (baseline). Cady et al. (30) found that the rate of change in impedance corresponds to the rate of bacterial growth. Consequently, the percent change in impedance was log transformed and plotted against time, and the slope of the exponential phase was calculated to determine the bacterial growth rate.

RNA preparation of *Salmonella* grown in M9 minimal medium with cecal content or glucose. *S.* Kentucky strain 1624-3N and *S.* Typhimurium strain 43R were used in DNA microarray studies. Both *Salmonella* strains were isolated from poultry. *S.* Kentucky 1624-3 represents one of three major PFGE types; it is less invasive than *S.* Typhimurium strain 43 in tissue culture but exhibits a competitive advantage over *S.* Typhimurium 43 *in vivo*, despite similar growth rates in minimal medium with cecal contents as the carbon and energy source. For preparation of initial inocula, bacteria were grown overnight aerobically at 37°C. The overnight culture was used to inoculate 100 ml of minimum M9 medium supplemented with 0.1% cecal content or 0.2% glucose. Bacterial growth in M9 minimal medium supplemented with 0.2% glucose as carbon source served as a growth control. Culture bottles for both experimental and control groups were placed in the same Campjar system (BD Diagnostic Systems, Franklin Lakes, NJ), which was flushed with a gas mixture of 6% CO_2, 6% O_2, 85% N_2, and 3% H_2. This experiment was repeated three different times for biological replicates needed for microarray analysis. In order to obtain abundant RNA needed for microarray analysis, pilot studies were conducted to monitor the growth of *S.* Kentucky 1624-3N and *S.* Typhimurium 43R in minimal M9 medium supplemented with either 0.2% glucose or 0.1% cecal content. Preliminary data revealed that both *S.* Kentucky and *S.* Typhimurium reached early stationary phase (optical density at 600 nm, 0.4; $\sim 1 \times 10^8$ CFU/ml) after 8 h in M9 minimal medium with 0.2% glucose. In contrast, both *Salmonella* serovars only reached a maximum cell density of 10^7 CFU/ml when grown in M9 minimal medium with 0.1% cecal content, regardless of the cell density used as the starting inoculum (range, 10^4 CFU/ml to 10^6 CFU/ml). In order to obtain a homogeneous, exponentially growing population from microaerophilic cultures in M9 minimal medium with 0.1% cecal content, a 10^5 CFU/ml starting inoculum of *Salmonella* was used. The low initial cell density allowed the bacteria to multiply for the same number of generations (10 generations) as did *Salmonella* grown in the control medium (M9 minimal medium with 0.2% glucose) starting at a cell density of 10^6 CFU/ml. Cells were harvested at the early stationary phase from 100 ml of culture at a cell density to produce a sufficient amount of RNA for microarray analysis.

Approximately 10^9 CFU of *Salmonella* were harvested and treated with a 0.1 volume of 95% ethanol, 5% acidic phenol (pH 4.3) to fix the cells and stabilize the RNA. Total RNA was extracted by using MasterPure Complete DNA and RNA purification kits (Epicentra, Madison, WI) and a High Pure RNA isolation kit (Roche). The samples were treated with a Turbo DNA-free kit twice (Ambion, Austin, TX). The elimination of contaminating DNA was confirmed via *Salmonella*-specific *hilA* PCR (see Table S1 in the supplemental material). The quality and integrity of the RNA was evaluated by agarose gel electrophoresis.

Molecular typing of *S.* Kentucky isolates by PFGE. Agarose plugs and PFGE conditions were used as previously described (31). Thiourea (1.65 mM) was added to the standard 0.5× Tris-borate-EDTA buffer (TBE), which is a modification to the CDC protocol (32). Electrophoresis was performed using a CHEF-DRII apparatus (Bio-Rad Laboratories, Hercules, CA) for a total of 20 h at 14°C using a voltage of 6 V/cm with pulse/switch times set at 2.2 to 54.2 s. Following electrophoresis, the gel was stained with ethidium bromide (1 mg/ml) for 30 min on a platform shaker and destained using ultrapure water for 60 min on the platform shaker. Gel images were captured using a digital camera (Molecular Imager Gel Doc XR system; Bio-Rad Laboratories) and saved as TIF files. A master database of *S.* Kentucky PFGE patterns was generated using BioNumerics (Applied Maths, Austin, TX). Comparisons were made between PFGE patterns by using the Dice coefficient (33) and unweighted-pair group method using average linkage (UPGMA) clustering.

Colony blots. A 96-well master plate was made for screening *Salmonella* isolates for selected virulence genes by DNA-DNA hybridization. Individual wells containing LB broth (BD Diagnostic Systems, Franklin Lakes, NJ) with 7% glycerol were inoculated with each isolate. The isolate number was recorded on a grid for later analysis. The last row of the master plate was used for positive and negative controls for each of the seven *Salmonella* virulence genes screened in this study. The plates were incubated overnight at 37°C and then stored at −80°C. This plate served as the template for inoculating LB agar plates (150 by 15 mm) with a flamed, 96-pin inoculator (Boekel Scientific, Feasterville, PA). LB agar plates were incubated at 37°C for 24 h, and then an 80- by 115-mm nylon membrane (Osmonics, Inc., Phoenix, AZ) was placed onto the plates to lift colonies from the agar surface. The nylon membranes were processed for DNA-DNA hybridizations as described by Sambrook et al. (34). DNA probes for the genes of interest were created by PCR with digoxigenin-labeled nucleotides as previously described (33) (see Table S1 in the supplemental material for a description of the primers and PCR conditions). A 10-μl PCR mixture was prepared with 0.5 μM each primer, 2.0 mM $MgCl_2$, 10 mM deoxynucleotide solution (Roche Diagnostics, Indianapolis, IN), 0.5 U *Taq* DNA polymerase (Roche Diagnostics), and 100 ng of DNA template. PCR primers and conditions are listed in Table S1. *Salmonella* strains SARB65, SARB66, SARB67 (35), and χ3968 served as positive

controls for the virulence genes screened, and *Escherichia coli* LE392 was used as a negative control. DNA-DNA hybridizations were performed as described by Sambrook et al. (34) with hybridizations and washes done at 68°C for all probes. Bound probe was detected with the an antidigoxigenin antibody–alkaline phosphatase conjugate as detailed for the Genius System (Roche Diagnostics).

DNA microarrays. Fluorescent labeling of RNA transcripts and hybridization conditions were as previously described (36). Genomic *Salmonella* DNA was used as a labeling and hybridization reference control. Microarrays were scanned on a ScanArray 5000 laser scanner (GSI Lumonics, Watertown, MA), and the data were analyzed using QuantArray v.2.01 software. Subtraction of background intensity from spot boundary signal intensities was performed to derive Cy3 and Cy5 median signal intensities. Differential gene expression was calculated from three independent experiments, and statistical significance was determined by utilizing the Significance Analysis of Microarrays (SAM) software package (Stanford University; http://statweb.stanford.edu/~tibs/SAM/). The data are expressed as means ± standard errors of the means (SEM). The data were analyzed by using a paired Student *t* test. To determine statistical significance between multiple comparisons, one-way analysis of variance (ANOVA) was performed, followed by a Bonferroni posttest. Data were considered statistically significant when P was <0.05.

qRT-PCR analysis. Quantitative reverse transcription-PCR (qRT-PCR) analysis was used to confirm differential expression of a set of selected genes between *S.* Typhimurium and *S.* Kentucky. One virulence gene and four metabolic genes were chosen from the microarray data set for qRT-PCR confirmation. qRT-PCR was performed with the QuantiTect SYBR green RT-PCR kit (Qiagen, Valencia, CA) and the MJ Research Chromo4 real-time 4-color 96-well PCR system (Bio-Rad). qPCR data were analyzed by the $2^{-\Delta\Delta CT}$ method (37). The housekeeping gene *rpoD* (σ^{70}) was used as an internal reference for data normalization (36). All experiments were done with three independent, biological replicates (samples collected on three different days) for statistical analysis.

Construction of *Salmonella* deletion mutants. The λ Red system was used to inactivate the gene operons/clusters of interest in *S.* Typhimurium 43R and *S.* Kentucky 1624-3N. *Salmonella* deletion mutants for *prpBCDE*, *narZYWV*, *mglBAC*, and *csgAB* were constructed as previously described (38). The mutations were transduced into a fresh *S.* Typhimurium SL1344 culture by using the phage P22 HT int (39). The deletions were introduced into *S.* Typhimurium 43 by transducing mutations from the SL1344 deletion mutant into the STM43 strain background via P22 HT int. Primers for the targeted gene deletions were designed using nucleotides sequences from the *S.* Typhimurium LT2 genome (GenBank accession number NC_003197.1). Through BLAST analysis we confirmed conservation of selected primer sequences in the *S.* Typhimurium SL1344 (GenBank accession number NC_016810.1), *S.* Kentucky CVM 29188 (GenBank accession number ABAK02000001.1), and *S.* Typhimurium STM43R (unpublished data) genomes. The specific sets of primers used are listed in Table S1 in the supplemental material. In each deletion mutant, the antibiotic resistance marker (chloramphenicol or kanamycin) was kept to facilitate the differentiation between the mutant and the corresponding parental strain in cecal colonization experiments. The deletion was confirmed by using the PCR primers used for the mutant constructions. The growth defects of Δ*prp* and Δ*mgl* mutants were confirmed phenotypically by using propionate and galactose as the single carbon source, respectively, in M9 minimal medium. Deletion of *csg* altered the colony morphology on Congo red agar from a rough, deep red colony type to a smooth pink morphology characteristic of curli-deficient strains (40).

Cell invasion assay. The human intestinal epithelial cell line Caco-2 and an avian epithelial cell line, BAT (41), were seeded in 12-well cell culture plates at a density of 10^4 cells/cm^2. The Caco-2 cells were cultured in medium containing minimal essential medium with Earle's salts (EMEM; Mediatech, Manassas, VA), 2 mM L-glutamine, 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 1.5 g/liter sodium bicarbonate, supplemented with 20% fetal bovine serum, and grown at 37°C in a 5% CO_2 incubator. BAT cells were cultured in EMEM supplemented with 5% fetal bovine serum, and chicken serum. The Caco-2 monolayers were maintained in the complete growth medium, whereas the BAT cells were maintained in medium containing 10% calf serum in place of fetal bovine serum. *S.* Kentucky 1624-3-3N and *S.* Typhimurium 43R were grown for 13 h at 37°C in 5 ml of LB broth without aeration. *E. coli* HB101 was included as a negative control. Ten microliters of bacterial cell suspension was transferred to tissue culture monolayers, resulting in a multiplicity of infection of 10 bacteria per epithelial cell. The tissue culture plates were then incubated for 120 min at 37°C, before adding gentamicin (100 μg/ml) and incubating plates for an additional hour at 37°C. The tissue culture monolayers were rinsed three times with phosphate-buffered saline (PBS) and lysed with LB broth plus 0.1% deoxycholic acid. Tenfold dilutions of the cell lysate were plated on LB agar and incubated overnight at 37°C.

***Salmonella* Kentucky and *S.* Typhimurium colonization and persistence in chickens.** A total of 120 1-day-old broiler chicks were acquired from a commercial hatchery. They were placed in HEPA-filtered, isolator units with wire mesh floors to prevent reinfection due to coprophagy, with a stock density similar to that in a commercial flock (30 birds/unit), and birds received diet and water *ad libitum* until 41 days of age. Papers lining the trays below mesh floors in isolator units were replaced the night before cecal droppings were collected for each group. Birds and their environment were culture negative for *Salmonella* on the day of placement of chicks in research units. At day 2 of age, animals were orally inoculated with either *S.* Typhimurium 43R (rifamycin resistant [Rif^r]; 64 μg/ml; 1.9×10^6 CFU), *S.* Kentucky 1624-3N (nalidixic acid resistant [Nal^r]; 64 μg/ml; 1.3×10^6 CFU), or *S.* Typhimurium 43R and *S.* Kentucky 1624-3N combined (1.1×10^6 and 1.6×10^6 CFU, respectively); negative-control birds were administered saline alone.

Chickens succumb to most *Salmonella* infections at 1 day of age but generally do not exhibit symptoms of illness at day 2 of age or older. Disease associated with *Salmonella* is generally rare in U.S. commercial chicken flocks. As the focus of this study was to examine *Salmonella* colonization, shedding, and persistence in chickens, we therefore chose to orally inoculate birds at 2 days of age.

Cecal droppings were collected at 1, 2, 3, 4, 18, 25, 32, and 39 days after inoculation. At 18, 25, 32, and 39 days following challenge, six birds per treatment group were euthanized and the small intestine, cecum, liver, and spleen were aseptically collected. Samples collected from the randomized birds were homogenized in pH 7.0 phosphate-buffered saline, and 10-fold serial dilutions were plated on XLT4 agar (BD Diagnostic Systems) containing the appropriate antibiotic and incubated overnight at 37°C. The University of Georgia Animal Care and Use and Procedures Committee approved all animal care protocols. Uninoculated, control animals were negative for *Salmonella* throughout the duration of the experiment.

***In vivo* competition of *Salmonella* deletion mutants with wild-type strains.** A chicken model for intestinal colonization was used to assess the relative fitness of the deletion mutants versus parental strains. The following competition groups were assayed: (i) *S.* Typhimurium 43R (STM43R; wild type) versus STM43Δ*prp*; (ii) STM43 versus STM43Δ*nar*; (iii) STM43 versus STM43Δ*mgl*; (iv) STM43 versus STM43Δ*csg*; (v) *S.* Kentucky 1624-3N (SKY; wild type) versus SKYΔ*prp*; (vi) SKY versus SKYΔ*nar*; (vii) SKY versus SKYΔ*mgl*; (viii) SKY versus SKYΔ*csg*. Procedures were carried out as described above. Equal numbers of the parental strain and the deletion mutant were mixed to give a final concentration of $\sim 3 \times 10^7$ CFU/ml. The selective antibiotic markers of parental and deletion mutant strains are listed in Table 1. Forty 1-day-old chicks were acquired from a commercial hatchery, placed in isolator units (5 birds/unit), and received diet and water *ad libitum* until 36 days of age. At 2 days of age, each bird orally received a 100-μl aliquot of mixtures of the wild-type strain and the deletion mutant (1:1 ratio of wild type and deletion mutant). For the first 9 days postinoculation, cecal droppings were collected daily. After day 9 postinoculation, cecal droppings were collected

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Description and/or phenotype[a]	Source or reference
S. enterica strains		
SKY 1624-3	*S. enterica* Kentucky isolated from poultry; deficient for *grvA*, *sseI*, *sodC1*, *sopE*, and the *spv* virulence plasmid	This study
SKY 1624-3N	Naladixic acid-resistant derivative of SKY 1624-3	This study
SKY Δ*csg*	SKY 1624-3N with deletion of *csgC* and *csgD* via λ Red targeted mutagenesis; Nal^r Kan^r; defective in red rough colony morphology on Congo red agar, associated with curli production	This study
SKY Δ*mgl*	SKY 1624-3N with deletion of *mglB* and *mglC* via λ Red targeted mutagenesis; Nal^r Cm^r; defective in growth on minimal M9 medium with galactose as carbon source	This study
SKY Δ*prp*	SKY 1624-3N with deletion of *prpB* and *prpE* via λ Red targeted mutagenesis; Nal^r Cm^r; defective in growth on minimal M9 medium with propionate as carbon/energy source	This study
SKY Δ*nar*	SKY 1624-3N with deletion of *narU* and *narV* via λ Red targeted mutagenesis; Nal^r Kan^r	This study
STM 43	*S. enterica* Typhimurium isolated from poultry; $grvA^+$ $sseI^+$ $sodC1^+$, *sopE* deficient; *spvB* virulence plasmid positive; Ap^r Cm^r	This study
STM 43R	Rifampin-resistant derivative of STM 43	This study
STM 43 Δ*csg*	STM 43R with deletion of *csgC* and *csgD* via λ Red targeted mutagenesis; Cm^r Kan^r; defective in red rough colony morphology on Congo red agar, associated with curli production	This study
STM 43 Δ*mgl*	STM 43R with deletion of *mglB* and *mglC* via λ Red targeted mutagenesis; Cm^r Kan^r; defective in growth on minimal M9 medium with galactose as carbon source	This study
STM 43 Δ*prp*	STM 43R with deletion of *prpB* and *prpE* via λ Red targeted mutagenesis; Cm^r Kan^r; defective in growth on minimal M9 medium with propionate as carbon/energy source	This study
STM 43 Δ*nar*	STM 43R with deletion of *narU* and *narV* via λ[ι] Red targeted mutagenesis; Cm^r Kan^r	This study
Plasmids		
pKD3	PCR template for amplifying *cat* cassette for λ Red targeted mutagenesis; oriRγ replicon; *cat* (Cm^r) *bla* (Amp^r)	38
pKD4	PCR template for amplifying *cat* cassette for λ Red targeted mutagenesis; oriRγ replicon; *kan* (Km^r) *bla* (Amp^r)	38
pKD20	Red recombinase expression plasmid; oriR101 temperature-sensitive replicon; *bla* (Amp^r)	38

[a] Amp^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Kan^r, kanamycin resistant; Nal^r, naladixic acid resistant; Rif^r, rifampin resistant.

every other day. Cecal droppings were pooled from chickens in each unit. The pooled cecal droppings were homogenized in PBS, and 10-fold dilutions were spotted on XLT4 plates containing appropriate antibiotics. The total number of *Salmonella* was recorded from the plate with the antibiotic to which both the parental strain and deletion mutant were commonly resistant (chloramphenicol, at 25 μg/ml for *S.* Typhimurium and nalidixic acid at 64 μg/ml for *S.* Kentucky). The cell number for the *S.* Typhimurium deletion mutant was determined from plates containing chloramphenicol (25 μg/ml) and kanamycin (50 μg/ml) (Table 1), while the *S.* Kentucky deletion mutant was enumerated on plates with nalidixic acid and chloramphenicol or kanamycin (Table 1). The cell density for the parental strain was calculated from the total *Salmonella* count minus the colony count for the deletion mutant. At day 29 and day 36, two chickens from each unit were euthanized. The ceca were removed from each bird and homogenized in PBS. The numbers of *Salmonella* from ceca were enumerated on XLT4 plates containing appropriate antibiotics as described above. The ratio of the mutant to parental strain recovered from cecal droppings was determined and compared to the input ratios to calculate the competitive indices (CI) for each experiment. CI values are presented so that a CI value of >1 indicates that the deletion mutant outcompeted the wild type, and a CI value of <1 indicates that the wild type outcompeted the deletion mutant. Due to inherent variations in the sampling method, only when the CI was consistently less than 0.1 (10-fold lower) and reduced colonization of mutants was further confirmed by enumeration of cecal contents on day 36 (termination) was a deletion mutant considered attenuated in its colonization of chickens.

Statistical analysis. The frequencies of distributions of virulence genes were analyzed by a chi-square test using PROC FREQ of SAS (version 9, 2005). The significant differences between birds challenged with either or both *S.* Kentucky 1624-3N and *S.* Typhimurium 43R were determined after data were $\log_{10}$ transformed using Tukey's test and the PROC GLM procedure of SAS (version 9). Student's *t* test was used to evaluate the growth kinetics, via PROC TTEST of SAS (version 9). In the invasion assay, the effects of cell line (Caco-2 and BAT cells) and bacterial strain (*S.* Typhimurium 43R and *S.* Kentucky 1624-3N) were statistically analyzed using Tukey's test via the PROC GLM procedure of SAS (version 9).

RESULTS

Genetic diversity and virulence gene distributions in *S.* Kentucky. PFGE was used to assess the genetic diversity of *S.* Kentucky. Of 68 isolates examined, eight distinct clusters (>85% similarity) were observed in *S.* Kentucky XbaI PFGE patterns (Fig. 1). Chicken isolates produced distinct PFGE patterns compared to human (AM1314 and AM19226) and bovine (K1877, K1915, K1976, and K1979) isolates. Within three PFGE clusters, clusters A to C, formed by chicken *S.* Kentucky isolates, 4 to 7 subtypes were identified within each cluster where PFGE patterns differed by 1 to 4 bands. The percentages of chicken isolates that fell into these clusters were 26%, 23%, and 51% for clusters A, B, and C, respectively. The *S.* Kentucky PFGE patterns belonging to clusters B and C were more similar to one another (>75% similarity) than patterns that comprised cluster A. Fifty-eight percent of chicken isolates were found to belong to 4 PFGE subtypes: B4, C1, C3, and C6.

Significant differences were observed in the distribution of virulence genes in *S.* Kentucky isolates compared to other *S. enterica* serovars isolated from the poultry. The fimbrial operons *saf* and *sef* were primarily found in *S.* Typhimurium and *S.* Enteritidis, respectively; *S.* Worthington isolates were positive by Southern analysis for *saf* (Table 2). All *S.* Kentucky isolates were negative for both *saf* and *sef* fimbrial operons. No significant differences were observed in the distributions of the fimbrial operons *lpf* and *stf* among *Salmonella* serovars isolated from poultry, with a high prevalence of both operons in *S.* Kentucky, 85% and 87%, respec-

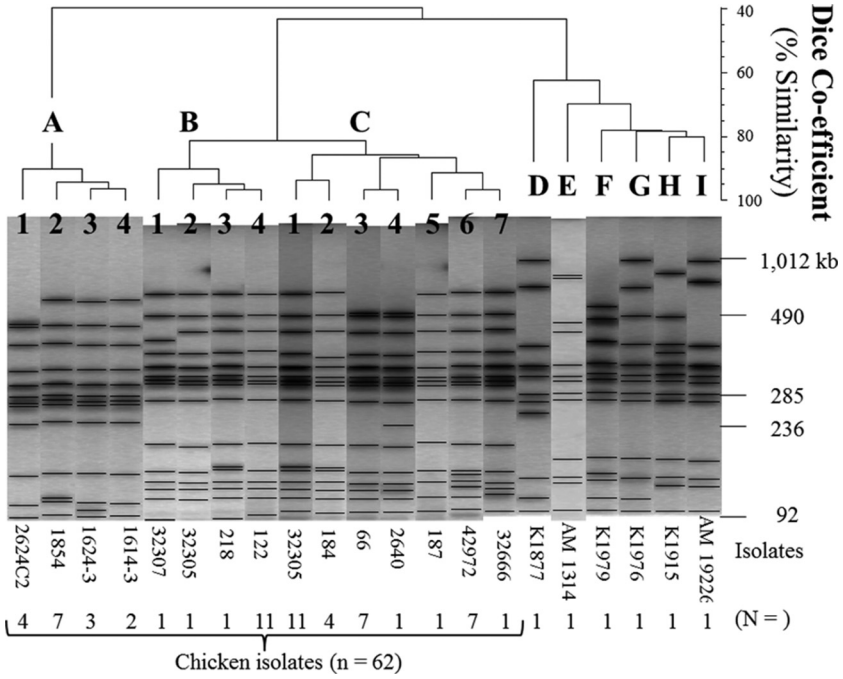

FIG 1 Cluster analysis of *S.* Kentucky PFGE patterns generated with the restriction enzyme XbaI. *S.* Kentucky PFGE patterns were compared using DNA pattern recognition software (BioNumerics; Applied Maths, Austin, TX). Levels of similarity were calculated using the band-based Dice similarity coefficient, and clustering of samples was performed using UPGMA. Nine major PFGE clusters, A to I (>85% similarity), were identified among 68 *S.* Kentucky isolates examined. Within PFGE clusters A, B, and C, additional PFGE subtypes, in which patterns differed by only 1 to 4 DNA fragments, were identified. The PFGE patterns for *S.* Kentucky isolates from chickens were distinctly different from patterns generated for isolates from humans or cattle.

tively. There was a nonrandom association of other virulence genes among *Salmonella* serovars isolated from the poultry, especially those genes linked to mobile genetic elements, plasmids, and phages (χ^2 test, $P < 0.005$). Poultry *S.* Kentucky isolates were negative for *spvC*, a marker for the *spv* virulence plasmid, and exhibited a lower prevalence of the phage-associated virulence genes *sopE* (31%), *grvA* (3%), *sodC1* (3%), and *sseI* (2%) compared to *Salmonella* Enteritidis (*sopE* [67%], *grvA* [0%], *sodC1* [100%], and *sseI* [100%]) and *S.* Typhimurium (*sopE* [67%], *grvA* [80%], *sodC1* [87%], and *sseI* [80%]).

***Salmonella* Kentucky persists longer in chickens than does *Salmonella* Typhimurium.** Cell invasion has been implicated as one important contributing factor for *Salmonella*'s colonization of its avian hosts (42). We identified two poultry *Salmonella* iso-

TABLE 2 Distribution of virulence genes among *Salmonella* serovars isolated from poultry, including the major chicken serovar, *S.* Kentucky

Salmonella serovar (*n*)	% of isolates with indicated virulence gene								
	Fimbrial genes					Phage-associated genes			
	lpfE	*saf*[a]	*stfA*	*sefC*[a]	Plasmid-carried *spvC*[a]	*sopE*[a]	*grvA*[a]	*sodC1*[a]	*sseI*[a]
Agona (5)	80	0	100	0	0	0	0	0	0
Enteritidis (9)	100	0	100	100	100	67	0	100	100
Hadar (1)	100	0	100	0	100	0	100	0	0
Heidelberg (80)	79	0	88	0	0	60	10	50	84
Infantis (3)	67	0	100	0	0	67	0	0	100
Kentucky (216)	85	0	87	0	0	31	3	3	2
Litchfield (14)	90	0	60	0	0	10	0	10	0
Mbandaka (3)	100	0	100	0	0	0	0	67	0
Montevideo (11)	0	0	0	0	9	0	36	0	0
Ohio (1)	100	0	100	0	0	0	0	100	0
Oranienberg (7)	100	0	100	0	0	29	0	0	0
Schwarzengrund (3)	0	0	0	0	0	67	0	0	0
Senftenberg (6)	83	0	100	0	0	50	0	0	0
Typhimurium (15)	93	80	93	0	93	67	80	87	80
Worthington (10)	100	100[b]	100	0	0	29	0	43	100

[a] The genes *saf*, *sefC*, *spvC*, *sopE*, *grvA*, *sodC1*, and *sseI* were found at statistically significantly different frequencies ($P < 0.05$; chi-square test) among the different serovars.
[b] Isolates were weakly positive for *saf* based on DNA-DNA hybridization results but were negative by PCR.

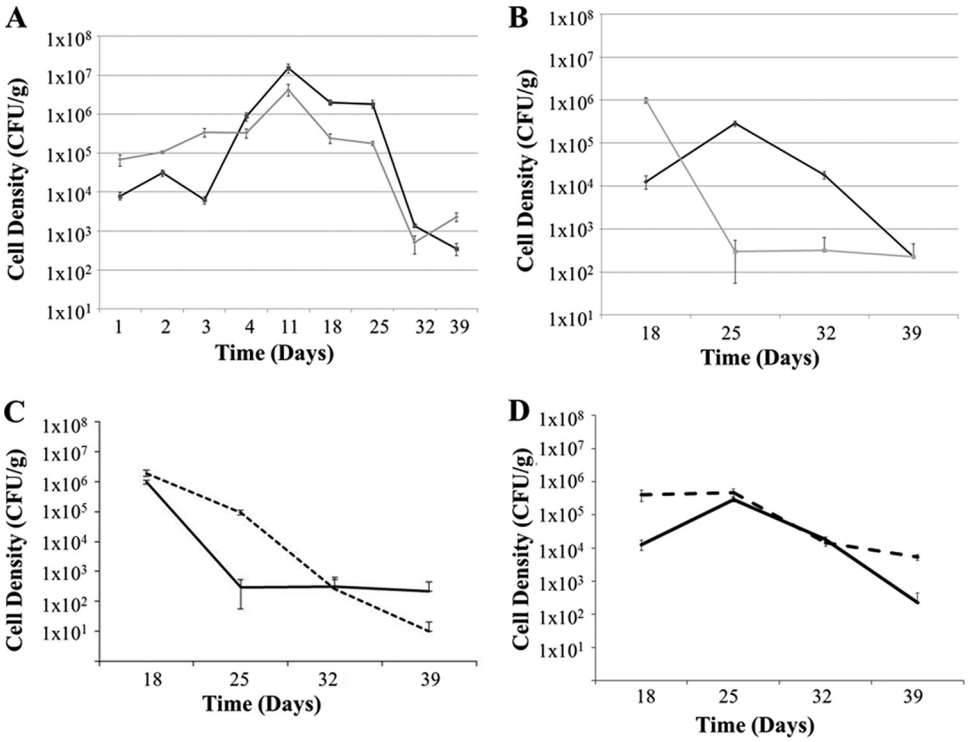

FIG 2 Competition between *Salmonella* Kentucky and *Salmonella* Typhimurium in chickens. Thirty 2-day-old chicks were orally inoculated with a 1:1 mix of *S.* Kentucky 1624-3N (black squares) and *S.* Typhimurium 43R (gray diamonds) (10^6 CFU) (A and B) or one of the two *Salmonella* strains alone: *S.* Typhimurium 43R (C; dashed line) or *S.* Kentucky 1624-3N (D; dashed line) and reared for 41 days in HEPA-filtered, isolator units with wire mesh floors with a stock density similar to that in a commercial flock. Rearing the birds in units with a wire mesh floor allowed for easy collection of fresh cecal droppings and prevented *Salmonella* reinfection that could occur due to coprophagous behavior of chickens. (A) Cecal droppings were collected on days 1, 2, 3, 4, 11, 18, 25, 32, and 39 following *Salmonella* challenge. (B to D) At the times indicated, birds ($n = 6$ per group) were sacrificed and ceca were aseptically removed and homogenized (tissue and content). The experiment was terminated when birds were 41 days of age. Samples were serially diluted 10-fold and plated on XLT4 plates with appropriate antibiotics for enumerating *S.* Kentucky and *S.* Typhimurium. (C) *S.* Typhimurium 43R colonization of the chicken ceca alone (dashed line) or in mixed infection (solid line) with *S.* Kentucky 1624-3N. (D) *S.* Kentucky 1624-3N colonization of the chicken ceca alone (dashed line) or in a mixed infection (solid line) with *S.* Typhimurium 43R.

lates, *S.* Kentucky 1624-3N and *S.* Typhimurium 43R, that were, respectively, deficient and proficient in cell invasion (see Table S2 in the supplemental material). We examined the colonization and persistence of these two *Salmonella* strains in chickens orally inoculated with *Salmonella* at 2 days of age. No significant differences were observed in fecal shedding of chickens infected with these two *Salmonella* strains, except at 3 days after challenge ($P < 0.001$) (Fig. 2A). *Salmonella* levels peaked at ~10^7 CFU/g on day 11 following challenge and persisted at high levels in cecal droppings (~10^6 CFU/g) until day 32, when levels dropped to 10^3 CFU/g on day 39. Because *Salmonella* typically colonizes the chicken cecum (43), we sought to determine if there were differences between the two *Salmonella* strains in the colonization of this organ. The *S.* Typhimurium cecal population continuously declined between 18 and 25 days postchallenge (Fig. 2B), showing a 4-$\log_{10}$ CFU/g decrease, while *S.* Kentucky numbers in the cecum increased during the same period. The *S.* Kentucky cecal population peaked at 25 days after challenge (5×10^5 CFU/g) and gradually declined over the following 2 weeks, until it reached levels of 100 CFU/g in the cecum. This rapid decline of *S.* Typhimurium in the chicken cecum appears to be attributable to the cocolonization and competition between the two serovars, since between 18 and 25 days postchallenge the *S.* Typhimurium decline was more gradual in the cecal environment of chickens challenged with *S.* Typhimurium alone (Fig. 2C).

Growth of *S.* Kentucky and *S.* Typhimurium in an *ex vivo* system using chicken cecal contents as the carbon and energy sources. Compared to the small intestine, the cecum is a nutritionally poor environment for bacteria like *Salmonella*, whose growth is dependent on free sugars, amino acids, or other available metabolizable compounds. Therefore, the growth of *Salmonella* is dependent on the microbial activity of primary degraders, like *Bacteroides* or the *Clostridiales*, organisms capable of liberating carbohydrates from indigestible fiber or host mucin and producing metabolizable fermentation end products. One possible explanation for the prevalence of *Salmonella* serovar Kentucky in poultry might be its competitive advantage in scavenging scarce nutrients available in this environment. If there were the case, *S.* Kentucky isolated from chickens would be expected to have a higher growth rate in intestinal contents of chickens than other *Salmonella* serovars also isolated from poultry. Sterilized cecal contents in minimal medium were used to determine growth rates. Of the *Salmonella* isolates selected, *S.* Kentucky ($n = 15$) and *S.* Typhimurium ($n = 16$) showed mean doubling times of 13.04 ± 0.32 h (range, 11.18 to 14.41 h) and 12.56 ± 0.20 h (range, 10.42 to 15.61 h), respectively (Table 3). No statistically significant differences were observed in the doubling times for these two serovars (Student's *t* test, $P = 0.06$). While there was genetic diversity within *S.* Kentucky isolates, as assessed by PFGE typing, no significant difference was seen in doubling times of isolates be-

TABLE 3 Growth of *S.* Kentucky and *S.* Typhimurium *in vitro* in medium containing 0.1% chicken cecal contents as carbon and energy source

Serovar and strain	Source/PFGE type	Doubling time[d] (h)
S. Typhimurium strains		
SARB65[a]	Reference strain	13.09 ± 0.18
SARB66[a]	Reference strain	13.18 ± 0.88
SARB67[a]	Reference strain	13.39 ± 0.12
SR11[b]	Reference strain	12.31 ± 0.40
SL1344[b]	Reference strain	12.99 ± 0.43
124831F[c]	Poultry isolate	11.19 ± 0.35
27868[c]	Poultry isolate	12.05 ± 0.06
176833 Heart[c]	Poultry isolate	10.42 ± 1.24
A196596B DS[c]	Poultry isolate	13.34 ± 1.43
196232YS[c]	Poultry isolate	10.42 ± 0.30
190691 Chick paper[c]	Poultry isolate	12.01 ± 0.82
181231CNT1A[c]	Poultry isolate	12.26 ± 0.08
452[c]	Poultry isolate	15.61 ± 1.96
285[c]	Poultry isolate	12.61 ± 0.24
920[c]	Poultry isolate	12.60 ± 0.16
43[c]	Poultry isolate	13.39 ± 0.33
Mean		12.56 ± 0.32
Range		10.42–15.61
S. Kentucky strains		
1624-3	Poultry isolate/A3	14.41 ± 0.40
122	Poultry isolate/B4	14.20 ± 1.96
100	Poultry isolate/B4	12.67 ± 0.05
165	Poultry isolate/B4	13.25 ± 0.25
140	Poultry isolate/B4	13.39 ± 0.09
189	Poultry isolate/B4	13.06 ± 0.07
185	Poultry isolate/C1	13.04 ± 0.22
32305-81	Poultry isolate/C2	12.61 ± 0.21
66	Poultry isolate/C3	13.58 ± 0.94
90	Poultry isolate/C3	13.13 ± 0.20
1617	Poultry isolate/C6	12.79 ± 0.20
32666	Poultry isolate/C7	11.18 ± 0.97
1277	Poultry isolate	12.90 ± 0.20
AM1314	Human isolate/E	13.45 ± 0.07
K1976	Bovine isolate/F	11.99 ± 0.26
Mean		13.04 ± 0.20
Range		11.18–14.41

[a] *Salmonella* reference collection B (SARB) (35).
[b] *S.* Typhimurium strain commonly used in virulence studies.
[c] Strain isolated from poultry.
[d] Doubling time values are means ± SEM.

longing to PFGE cluster B versus C (Student's *t* test, $P = 0.18$; doubling times of 13.34 and 12.72 h for clusters B and C, respectively). There was more variability within the subtype B4 PFGE cluster (doubling times, 12.67 to 14.20 h) and similarly, differences in growth rates were also apparent within *S.* Typhimurium strains. Comparisons in growth of *S.* Typhimurium poultry isolates versus reference strains revealed no significant differences for *S.* Typhimurium (Student's *t* test, $P = 0.21$; doubling times of 12.31 and 13.01 h for poultry and reference strains, respectively).

Comparative gene expression profiles of *S.* Kentucky and *S.* Typhimurium strains grown in chicken cecal contents. The *in vivo* colonization data, along with epidemiological data, revealed that *Salmonella* Kentucky has an advantage over other *Salmonella* serovars in chickens, either due to a metabolic advantage in utilization of carbon or energy sources present in the chicken cecum or due to the differential expression of a colonization factor(s) that enhances its persistence in the chicken cecum. In order to determine what core *Salmonella* genes are differentially expressed in response to the chicken cecal environment, a comparative transcriptomic analysis of *S.* Kentucky 1624-3N and *S.* Typhimurium 43R was performed under conditions designed to mimic the chicken cecum. To identify differential gene expression, a DNA microarray that included 4,587 of the 4,716 predicted open reading frames of the *S.* Typhimurium LT2 genome was used. In each of three replicate experiments, *Salmonella* was cultivated in minimal medium supplemented with chicken cecal contents, and the growth rate was compared with that of cells grown in minimal medium supplemented with glucose. With a cutoff of a ≥1.5-fold change for the ratio for cells grown on cecal contents versus glucose ($P < 0.05$), 94 genes were differentially expressed in *S.* Typhimurium 43R when grown in chicken cecal contents. Among these 94 genes, 62 genes were activated and 32 genes were repressed. In *S.* Kentucky 1624-3N, 254 genes were differentially expressed in response to the chicken cecal contents, with 173 genes activated and 81 genes repressed. To identify the strain-dependent transcriptomic differences, the growth profile of *S.* Kentucky 1624-3N (SKY) was compared to that of *S.* Typhimurium 43R (STM) in medium containing cecal contents as the carbon source, as well as medium with glucose as the carbon source. For each carbon source used, a SKY/STM ratio of ≥1.5-fold ($P < 0.05$) was considered significant differential expression between the two isolates. We defined the *S.* Kentucky-specific cecal content-activated genes as those having a ratio for growth in cecal contents versus glucose of >1.5-fold ($P < 0.05$) compared to *S.* Kentucky 1624-3N and a SKY/STM ratio of >1.5-fold ($P < 0.05$) for growth in medium with cecal contents. It should be noted that the microarray used in this study was constructed based on the *S.* Typhimurium strain LT2. Any genes unique to the *S.* Kentucky serovar or highly divergent from LT2 would not be identified using this system.

qRT-PCR analysis for 9 genes for each serotype was carried out to validate the microarray results. Log_2-transformed expression values for 18 genes correlated well with the microarray expression data ($R^2 = 0.83$) (see Fig. S1 in the supplemental material). For several genes, the change detected by qRT-PCR appeared to be higher than that detected by microarray (Table 4).

Transcriptional response of *S.* Kentucky to cecal content. Several genes and operons associated with catabolic pathways were differentially expressed in medium containing cecal contents, accounting for 31% of *S.* Kentucky's response to growth conditions, and approximately half of these genes encoded enzymes and cognate transporters for carbohydrate, polyol, and carboxylate catabolism (Fig. 3; see also Table S3 in the supplemental material). The genes encoding enzymes of the tricarboxylic acid (TCA) cycle and the glyoxylate pathways figured prominently in *Salmonella*'s metabolic response, along with genes for transporters and enzymes involved in funneling organic acids (fatty acids [*fadABCEH*] or proprionate [*prpB-E*]) and amino acids (arginine [*argT* and *astCADB*], 4-hydroxyphenylacetate [*hpaGEDF*], or γ-aminobutyric acid [*ygaFgabDTP*]) into this central pathway (Fig. 4A; see also Table S1 in the supplemental material). In addition, there was also expression of genes involved in anaerobic respiration, specifically, the nitrate reductase Z complex (NarV-Z), dehydrogenases (pyruvate [*poxB*], lactaldehyde [*aldB*], and

TABLE 4 Differential regulation of core *Salmonella* genes in *S.* Kentucky 1624-3N and *S.* Typhimurium 43R, based on qRT-PCR results

Target gene	Relative expression[a] in:	
	S. Kentucky	*S.* Typhimurium
argI[b]	2.46 ± 1.20	−0.76 ± 2.46
csg[b]	6.66 ± 4.64	0.86 ± 0.75
mglA[b]	8.21 ± 1.24	6.76 ± 0.48
narZ[b]	6.83 ± 1.93	4.34 ± 2.91
prpD[b]	9.7 ± 0.91	6.07 ± 5.03
hilA[c]	0.1 ± 0.33	−0.77 ± 0.06
luxS[c]	0.81 ± 0.35	−0.03 ± 0.16
phoP[c]	−0.46 ± 0.38	−0.39 ± 0.51
relA[c]	−0.24 ± 1.56	−0.81 ± 0.34

[a] Relative expression level (mean ± standard deviation) of the target gene with respect to housekeeping gene *rpoD*, as detected by real-time RT-PCR and calculated using the $2^{-\Delta\Delta CT}$ method (37), assuming perfect amplification efficiency. Relative expression levels were converted to $\log_2$ values. Averages of $\log_2$-transformed expression values were taken across biological replications, and the standard deviations were calculated.
[b] A *Salmonella* gene that was identified by microarray and was upregulated in *S.* Kentucky grown in minimal medium with cecal contents as a carbon and energy source (compared to growth with glucose) and differentially regulated compared to *S.* Typhimurium grown under similar conditions.
[c] A *Salmonella* core gene that was not differentially regulated under the different growth conditions (cecal content versus glucose as carbon/energy source) or between strains.

formate dehydrogenase-O [*fdoGH*]), and transporters (D-alanine [*cycA*]) (Fig. 4B; see also Table S3). In contrast, several genes associated with anaerobic respiration involving formate dehydrogenase (*fdhF*), hydrogenases (*hybA-D*, *hycA-H*,and *hypABDF*), and dimethyl sulfide reductase (*dmsAB*) (see Table S3) were repressed under these growth conditions. There was also no expression detected for the genes involved in anabolic pathways associated with amino acid and nucleotide synthesis (Fig. 3; see also Table S2 in the supplemental material) when *S.* Kentucky was grown in cecal contents versus glucose. Growth of *S.* Kentucky in cecal contents, compared to that in glucose, also led to expression of genes involved in manganese transport (*sitA-C*), iron transport (*iroN*), and storage (*ftnB* and *bfr*).

A significant stress response was observed for *S.* Kentucky grown in cecal contents. These genes were categorically associated with the stress response to high osmolarity (*osmCEY*, *otsAB*, and *yehYZ*), oxidative stress (*dps*, *katE*, STM1731, *msrA*, *grxB*, *yfcG*, *yajG*, *yibf*, STM4267, *ggt*, and *sufBDS*), and the stationary phase/carbon starvation (*rpoS*, *rsd*, *wraB*, *cstA*, and *csiE*). *rpoS* appeared to be the central regulator for *S.* Kentucky's response to growth in cecal contents.

While several catabolic pathways appear to be activated in *S.* Kentucky when grown in cecal contents versus glucose, anabolic pathways were repressed under these growth conditions. Specifically, anabolic pathways responsible for nucleotide and amino acid biosynthesis were repressed.

Eighty-three percent ($n = 249$) of core *Salmonella* genes described were differentially expressed in *S.* Kentucky when grown in cecal contents versus glucose and were compared to the *S.* Typhimurium strain. The propionate catabolism operons (*prpB* and *prpCDE*) exhibited a greater change in *S.* Kentucky than in *S.* Typhimurium in response to chicken cecal contents (see Table S3 in the supplemental material). In addition, several *S.* Kentucky-specific cecal content-activated genes were identified in this study. *otsAB*, encoding enzymes that catalyze trehalose biosynthesis, was induced to significantly higher levels in *S.* Kentucky 1624-3N than in *S.* Typhimurium 43R (SKY/STM ratios in cecal content were 1.77 and 2.07 for *otsA* and *otsB*, respectively; $P < 0.05$), suggesting that these two strains respond differently to the environmental stress encountered in the cecal contents. A significantly higher expression level was also observed for a central metabolic gene, *tktB* (which is involved in the pentose phosphate pathway) in *S.* Kentucky 1624-3N than in *S.* Typhimurium 43R (SKY/STM ratio in cecal contents, 1.86; $P < 0.05$) (see Table S3).

The expression of curli operons was significantly higher in *S.* Kentucky strain 1624-3N grown in cecal contents than in *S.* Typhimurium strain 43R. In *S.* Kentucky strain 1624-3N, all genes except *csgC* within the operons exhibited a range of 2.1- to 3.67-fold changes, whereas a range of −0.15- to 0.71-fold changes were observed for *S.* Typhimurium (see Table S2 in the supplemental material). A closer examination of *csgC* expression in the microarray data set revealed that the average intensity of the *csgC* signal was less than 9, suggesting that the *csgC* signal is weak on LT2 microarray slides (data not shown).

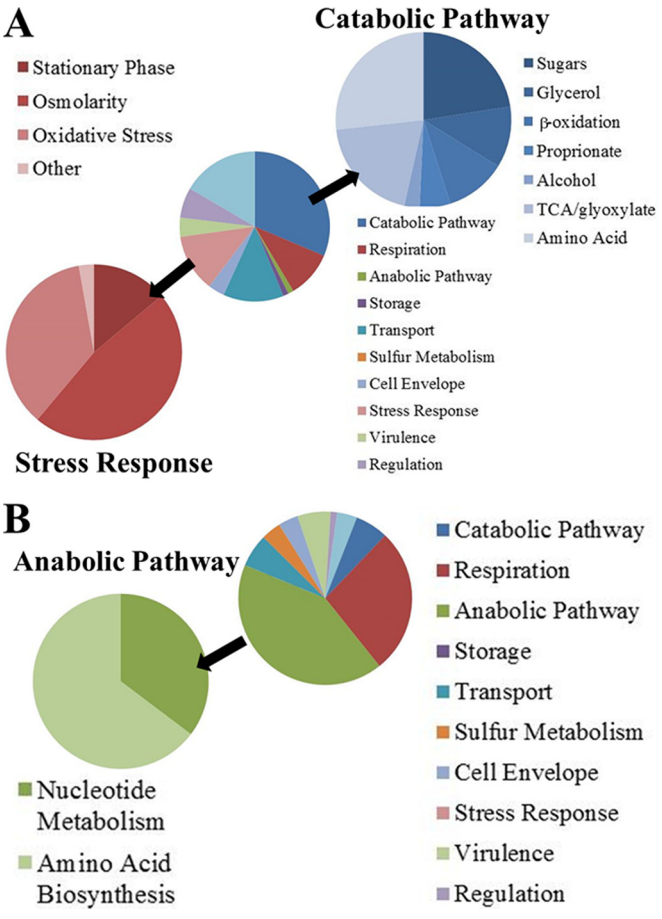

FIG 3 *S.* Kentucky transcriptional response to growth in M9 medium containing cecal contents as a carbon and energy source. *S.* Kentucky 1624-3N was grown in M9 medium with 0.1% glucose or cecal contents, total RNA was extracted from early-stationary-phase cultures, and a microarray was used to assess global gene expression. Genes (see Table S3 in the supplemental material) that were expressed (A) or repressed (B) in response to growth in medium with cecal contents versus medium with glucose, for both *Salmonella* serovars, were categorized based on function. A more detailed description of specific categories is presented for genes related to catabolic pathways (A; blue), the stress response (A; red), and anabolic pathways (B; green).

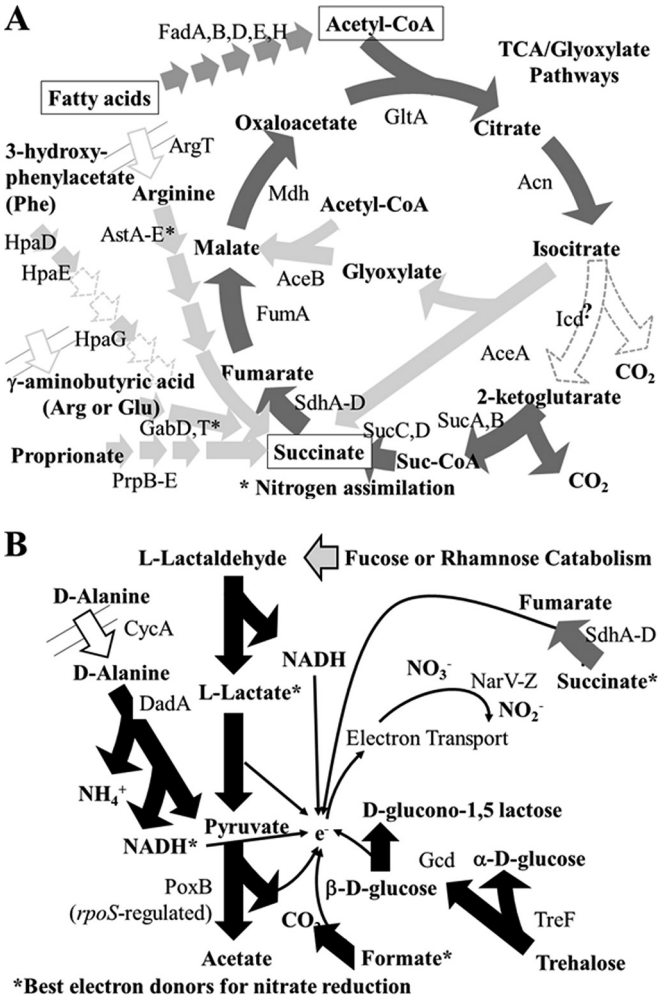

FIG 4 *S. enterica* Kentucky growth in M9 medium containing cecal contents as a carbon and energy source affects TCA/glyoxylate pathways (A) and anaerobic respiration (B). *Salmonella* genes expressed in response to growth in M9 medium with cecal contents versus glucose were mapped to metabolic pathways and individual steps within each pathway. Each solid arrow represents a gene, and presumably also an enzyme, that is expressed for that step when cells are grown in M9 medium with cecal contents. The arrows created with dashed lines represent pathway steps where gene expression was not detected. Transporters (arrows drawn across parallel lines) and peripheral pathways that feed into the TCA cycle (A) are also presented. Asterisks indicate several dehydrogenases and likely substrates identified that complement reductases expressed when *Salmonella* is grown in the presence of cecal contents (B).

Several genes associated with flagellar synthesis and chemotaxis were expressed at higher levels in *S.* Kentucky 1624-3N than in *S.* Typhimurium 43R, regardless of the carbon source (cecal content versus glucose). Three class III flagellar genes (*cheM*, *tsr*, STM2314, *fliC* and *fliD*) had higher transcript levels in *S.* Kentucky 1624-3N than in *S.* Typhimurium 43R. This regulation seems to be strain specific, since the *S.* Kentucky strain exhibited a higher expression level of these genes following growth in either cecal contents or glucose compared to *S.* Typhimurium strain 43R (SKY/STM ratio, >1.5; $P < 0.05$) (see Table S3 in the supplemental material). Furthermore, the expression levels of *cheM*, *fliC*, *fliD*, *tsr*, and STM2314 were not altered by growth in cecal contents for either *S.* Kentucky or *S.* Typhimurium strains ($P > 0.05$).

The role of *in vitro* cecal content-activated operons in *Salmonella* colonization and persistence *in vivo*. In order to determine the contribution of the cecal content-activated genes to cecal colonization, we orally dosed chickens with a mixture of the deletion mutant and its parental strain. This approach allowed us to directly assess the contribution of the selected genes by minimizing the animal-to-animal variation. In this competitive infection model, the cecal colonization of deletion mutants and the wild-type strain was tracked for 36 days. In the *S.* Typhimurium 43R background, we did not observe any clear trend or disadvantage for any of the deletion mutants. Cecal colonization by the Δ*mgl* mutant was lower than that of the wild type after day 15 for *S.* Typhimurium 43R. However, the competitive index was >0.1. In contrast, the *S.* Kentucky 1624-3N Δ*mgl* mutant CI was consistently 1 $\log_{10}$ lower (competitive index, <0.1) than that of its wild type after 15 days postinfection (Fig. 5B). At the end of the challenge (day 36), the *S.* Kentucky 1624-3N Δ*mgl* mutant dropped below our detection limit (<100 CFU/g), while the wild type persisted at 10^3 CFU/g. *S.* Kentucky 1624-3N Δ*csg* remained consistently lower than the wild type after day 21 postinfection (Fig. 5D). The deletion mutant was 1 to 2 $\log_{10}$ lower than the wild type at days 30, 33, and 36 postinfection. At the end of challenge (day 36), the *S.* Kentucky 1624-3N Δ*csg* mutant dropped below our detection limit (<100 CFU/g), while the wild type persisted at $\sim 10^3$ CFU/g. The *S.* Kentucky 1624-3N Δ*nar* and Δ*prp* mutants fluctuated in levels relative to the wild type from day 3 to day 36 (Fig. 5A and C). In general, the *S.* Typhimurium mutant and wild-type strains were transient in their colonization beyond day 15, often falling below the level of detection (<100 CFU/g) (Fig. 5A to D).

DISCUSSION

Salmonella serovars routinely isolated from chickens in the United States are markedly different from serovars isolated from human cases. Why *S.* Kentucky is commonly found in poultry is not clear; however, its prevalence in U.S. poultry does not appear to be attributable to the introduction of a clonal strain. The dissonance between the *S.* Kentucky prevalence in poultry and the disease incidence in humans may be attributable to the distribution of phage-associated virulence genes *grvA*, *sopE*, *sseI*, and *sodCI* and the *spvB* virulence plasmid in *S. enterica* serovars. Although *S.* Kentucky possesses the common *Salmonella* fimbrial genes *lpf* and *stf*, it lacks the Sef fimbriae of poultry-adapted serovars *S.* Enteritidis, *S.* Gallinarum, and *S.* Pullorum (16). While additional differences in fimbrial gene distribution may further explain *S.* Kentucky's reduced virulence in humans, it does not explain its advantage in colonizing chickens compared to poultry-adapted serovars, like *S.* Enteritidis.

An alternative explanation to *S.* Kentucky's emergence as the dominant serovar in poultry is selection of a serovar uniquely adapted to growth in the chicken intestine. A *Salmonella* serovar with a higher growth rate in the chicken cecum would be expected to outcompete other serovars. However, there was no apparent difference in the growth rates of *Salmonella* serovars *S.* Kentucky and *S.* Typhimurium grown in M9 minimal medium with chicken cecal contents as the carbon and energy source. Despite similar growth rates *in vitro*, *S.* Kentucky exhibited an advantage over *S.* Typhimurium in its persistence in chickens colonized with both serovars. While there were no differences observed in the fecal shedding of either *Salmonella* strain, *S.* Kentucky persisted longer in the cecum and at higher cell densities than *S.* Typhimurium. *S.*

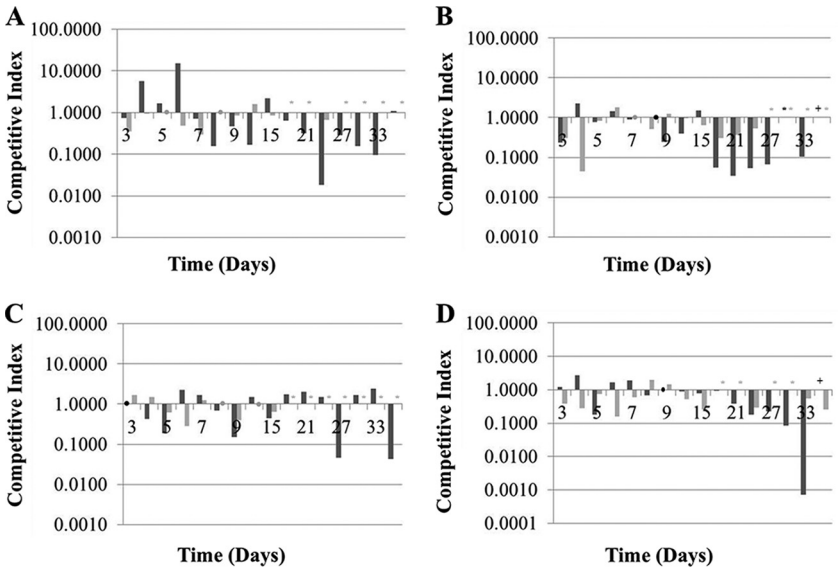

FIG 5 The contribution of propionate utilization, methyl-galactose transport, nitrate reductase, and curli to *S.* Kentucky and *S.* Typhimurium colonization and persistence in chickens. Core *S.* Typhimurium LT2 *prp* (propionate utilization), *mgl* (methyl-galactose transport), *nar* (nitrate reductase), and *csg* (curli) operons were upregulated >2.5-fold in *Salmonella* Kentucky grown in cecal contents, compared to salmonellae grown in glucose. These genes were deleted in *Salmonella* strains *S.* Kentucky 1624-3N and *S.* Typhimurium 43R by using λ Red. Two-day-old chickens (5 birds per competition group) were orally administered a suspension of 10^6 bacteria containing a 1:1 ratio of a deletion mutant versus wild-type *S.* Kentucky (dark gray) or *S.* Typhimurium (light gray). Cecal droppings were collected daily from chickens for 17 days. The impact of deletions of *prp* (A), *mgl* (B), *nar* (C), and *csg* (D) on *Salmonella* colonization and persistence in chickens were determined from based on CIs. ○, mutant:wild type CI ratio of 1.0, i.e., no effect was observed; *, *Salmonella* levels for the mutant and wild type were below the limit of detection (<100 CFU/g); +, the *Salmonella* mutant was below the limit of detection (<100 CFU/g) while the wild-type strain was present in cecal droppings at ~10^3 CFU/g.

Kentucky's advantage over *S.* Typhimurium in its persistence in the chicken cecum appeared to be attributable, in part, to competition or inhibition. The cell invasion-deficient *sseI*-negative *sopE*-negative *S.* Kentucky strain was not at a disadvantage in colonizing chickens compared to a cell invasion-proficient *S.* Typhimurium strain.

The differences in the persistence of these two poultry *Salmonella* strains may also be attributed to their unique response to the environment of the chicken cecum. We used transcriptomics to measure *Salmonella* strain responses to growth in medium containing either cecal contents or glucose as a carbon and energy source and an environment that simulated the anoxic environment of the cecum. Whole-genome expression profiles identified 160 core *Salmonella* genes that were differentially regulated in *S.* Kentucky compared to *S.* Typhimurium when cells were grown in cecal contents. Twenty-two percent of these differentially expressed genes have been reported to be regulated by the stationary-phase sigma factor *rpoS* (σ^s) (44). These *rpoS*-regulated genes included those typically associated with the stress response as well as others involved in metabolism. Curli also figured prominently in the expression profile of *S.* Kentucky; this expression is controlled by σ^s in *Salmonella* and *Escherichia coli* (40). Of the genes differentially expressed in *Salmonella* cells grown in cecal contents versus glucose, we chose to focus on 4 gene operons that were either activated only in *S.* Kentucky (*csg*) or expressed in both strains (*mgl*, *nar*, and *prp*).

mglCA and *mglB* encode high-affinity ABC transporters for galactose uptake. This galactose transporter appears to be essential for *S.* Typhimurium colonization of chickens (45, 46). The genes necessary for galactose uptake and utilization are also expressed by *S.* Typhimurium in the murine cecal lumen (47), and *mglB* promoter activity is particularly high in the mucus layer of the intestine (48). The elevated expression of *mglAB* may indicate the availability of galactose for bacterial growth in the intestine. Our analysis of *in vivo* competitive growth of the wild type versus Δ*mgl* mutant confirmed the importance of this galactose transporter in *S.* Kentucky colonization of broiler chickens and the availability of galactose in the cecum. The impact of the Δ*mgl* mutation on *Salmonella* colonization was only observed late in infection, which may have reflected *Salmonella*'s response to changes in the composition of the intestinal microbiome as the animal develops (49). While to date the carbon sources available for *Salmonella* in the cecum are largely unknown, there is indirect evidence for galactose as one major nutrient that can support *Salmonella* growth.

In our study, we detected higher expression levels of TCA/glyoxylate pathway genes in both *S.* Kentucky and *S.* Typhimurium in response to the cecal environment. Given their central role to metabolism, especially with regard to catabolism of sugars, amino acids, fatty acids, and fermentation end products, high-level expression of these central metabolic genes is essential for *Salmonella* growth in its animal host (45, 50). Other gene operons that were significantly induced included those for propionate catabolism (*prpB* and *prpCDE*) and anaerobic respiration using nitrate as a terminal electron acceptor (*narZYWV*). The *prpBCDE* locus encodes the propionate-degrading enzymes. Harvey et al. reported that the *prpC*, *prpD*, and *prpE* genes of an *S.* Typhimurium strain were also upregulated during colonization of the cecal lumen in 1-day-old chicks, but the *prpE* mutant showed no reduction in colonization. Those authors suggested that propionate catabolism may not be essential due to the other energy sources available to *Salmonella* within the lumen (51). We found these two gene clusters were induced in both serovars but to a significantly

higher level in *S.* Kentucky. Short-chain fatty acids (SCFA), including acetate, propionate, and butyrate, are produced by resident bacteria in the gastrointestinal tract. In chickens, the production of SCFA reaches and stabilizes at optimal concentrations for pathogen exclusion (acetate at 70 μmol/g; propionate at 8 μmol/g; butyrate at 24 μmol/g) after 15 days (52). The *S.* Kentucky Δ*prp* strain was intermittently reduced in cecal colonization after day 18. While the CI was reduced, it was intermittent and never less than 0.1. In contrast, no consistent difference was observed between wild-type *S.* Typhimurium and the Δ*prp* strain in the colonization of chickens in this study. The results from the *in vivo* competitive model suggest that propionate catabolism probably makes no major contribution to *Salmonella* colonization in chickens, consistent with the results reported by Harvey et al. (51).

As the TCA/glyoxylate pathway is fully expressed in the chicken cecum, anaerobic respiration is essential for the bacterial cell to realize the full energy potential of the various metabolites that are fed into this central pathway. Therefore, alternate electron acceptors, such as nitrite, nitrate, and fumarate, become essential for bacterial growth. The higher expression levels of nitrate reductase operons in *S.* Kentucky suggest that it may have a metabolic advantage in the chicken cecum. In our competition study, the *S.* Typhimurium Δ*narZYWV* strain colonized as well as the wild type, indicating that the nitrate reductase encoded by *narZYWZ* is not essential for colonization of the chicken intestine. Similarly, the nitrate reductase encoded by *narZYWZ* does not significantly contribute to colonization by *S.* Kentucky. As *Salmonella* has two nitrate reductases, it may be necessary that both operons be deleted before an effect on colonization can be observed.

While taking metabolic advantage of the intestinal milieu may contribute to *S.* Kentucky's competitive fitness during the process of cecal colonization, some nonmetabolic factors may be equally important for *Salmonella*'s successful colonization of the chicken gastrointestinal tract. The most prominent difference, at the transcriptional level, was observed in the curli operon between *S.* Kentucky and *S.* Typhimurium isolates. The two operons *csgDEFG* and *csgBAC* are responsible for the biogenesis of the aggregative fibers (curli), which is a major component of the multicellular morphotype involved in biofilm formation on biotic or abiotic surfaces and cell clumping in liquid culture (40). Curli are thought to be involved in attachment of *Salmonella* and *E. coli* to the mucosa (46). Upregulation of *csgB* and *csgA* was observed during cecal colonization by *S.* Typhimurium in the study by Harvey et al. (51). However, in their 1-day-old chick model, curli production appeared to play a small role in colonization by *S.* Typhimurium. For *S.* Kentucky, deletion of the *csg* operon resulted in a consistently significant reduction of colonization after day 18, confirming the role of curli production in long-term colonization. However, *csg* deletion had no effect on the *S.* Typhimurium colonization of chickens. The differential expression of the curli operons suggests behavioral differences may exist between these two *Salmonella* strains under the condition mimicking nutrient availability in the chicken cecum. Increased expression of CsgD is especially significant in the cecum, as this protein has been shown to stabilize σ^S (53). White et al. reported that the expression of *rpoS*-regulated genes (*otsAB*, *sdhD*, *fumC*, *mdh*, and *sucABCD*) was correlated with the level of *csgD* (54). Similarly, in our study, the expression levels of several *rpoS*-regulated genes were also induced to a higher level by cecal contents for *S.* Kentucky than in *S.* Typhimurium.

In summary, we have shown that *S.* Kentucky presents an adaptive gene expression profile under conditions mimicking the chicken cecum. A higher expression level of *rpoS*-regulated genes in *S.* Kentucky than *S.* Typhimurium may partially explain why *S.* Kentucky has become a successful colonizer of chickens. Our competitive colonization data further support the role of galactose and curli production in colonization of *S.* Kentucky. Future comparative transcriptomic analysis on more phylogenetically diverse *Salmonella* strains will define a common cecal gene expression profile shared with *S.* Kentucky strains. Our data provide a starting point for formulating new hypotheses to improve our understanding of the mechanisms that confer a competitive advantage for poultry-adapted *Salmonella* serovars over other serovars in human disease. Genetic and functional analysis of *Salmonella* core genes differentially regulated in *S.* Kentucky, and those *S.* Kentucky-specific genes recently identified from its annotated genome, will greatly improve our understanding of the molecular basis underlying the success of *S.* Kentucky as a colonizer in chickens.

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