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Virulence and Metabolic Characteristics of *Salmonella enterica* Serovar Enteritidis Strains with Different *sefD* Variants in Hens

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***Salmonella enterica* serovar Enteritidis is one of a few *Salmonella enterica* serotypes that has SEF14 fimbriae encoded by the *sef* operon, which consists of 4 cotranscribed genes, *sefABCD*, regulated by *sefR*. A parental strain was used to construct a *sefD* mutant and its complement, and all 3 strains were compared for gene expression, metabolic properties, and virulence characteristics in hens. Transcription of *sefD* by wild type was suppressed at 42°C and absent for the mutant under conditions where the complemented mutant had 10^3 times higher transcription. Growth of the complemented mutant was restricted in comparison to that of the mutant and wild type. Hens infected with the wild type and mutant showed decreased blood calcium and egg production, but infection with the complemented mutant did not. Thus, the absence of *sefD* correlated with increased metabolic capacity and enhanced virulence of the pathogen. These results suggest that any contribution that *sefD* makes to egg contamination is either unknown or would be limited to early transmission from the environment to the host. Absence of *sefD*, either through mutation or by suppression of transcription at the body temperature of the host, may contribute to the virulence of *Salmonella enterica* by facilitating growth on a wide range of metabolites.**

Salmonella enterica serovar Enteritidis is the only serotype of approximately 2,600 that contaminates eggs produced by otherwise healthy hens in a manner that persistently results in foodborne illness (7, 19, 25). The association of *S.* Enteritidis with eggs and egg products may have evolved from a homologous recombination event resulting in a hybrid chromosome that quickly split into two major lineages (15, 21, 23).

Recently, 250 single nucleotide polymorphisms (SNPs) that differentiate virulence phenotypes of *S.* Enteritidis belonging to the same phage type were identified (14). The gene *sefD* (SEN4250; NCBI assay identifier ss153929231) was 1 of 16 within the database that had an altered open reading frame (ORF). *sefD* is part of an operon, *sefABCD*, under the regulation of *sefR* that produces the entire SEF14 fimbria with a molecular weight of approximately 14,400 (4, 5, 11, 28). The majority of *Salmonella enterica* serotypes with characterized genomes do not have a complete *sefD*. The intact operon appears to be restricted to *Salmonella enterica* serotypes belonging to group D1 and to the closely related serotype *S.* Paratyphi A. *S.* Pullorum varies in having a complete *sefD*, and it is reported to be a pseudogene in *S.* Gallinarum (31). *S.* Enteritidis, *S.* Gallinarum, and *S.* Pullorum have all been shown to share the same deletion of a single adenosine residue in an 8-bp poly(A) region that begins at base pair 189 out of 444 (31). *S.* Paratyphi A had two adenosines deleted from the same poly(A) region.

The function of SefD is debatable, as separate studies hypothesize that it is the major fimbrin unit of a distinct fimbria (6), the minor subunit adhesin tip of SEF14 (12), or a minor fimbrin subunit of SEF14 that is inserted at intervals and acts as a branching molecule (3). SefD is required for proper assembly of SEF14, because *sefD* mutants lack SEF14 fimbriae altogether (3, 12). The impact of SefD on the pathobiology of *S.* Enteritidis is not evident, in part because analysis of SEF14 function is most often investigated as loss of the entire operon or mutation of the structural subunit *sefA*. For example, loss of SEF14 did not alter adherence in an *in vitro* gut explant assay (1, 26), internal organ invasion in chickens (26), invasion of cultured epithelial cells (9, 22), pathobiology in chicks (10, 27), or virulence in mice (22). SEF14 may mediate adherence to inanimate objects (29) and contribute to cecal colonization and fecal shedding (26). SefD appears to be essential for efficient uptake or survival of *S.* Enteritidis in mammalian macrophages (12). Increased knowledge of the impact of SefD on the hen will help determine if vaccination should include it as an immunogen or if it is irrelevant to the immunology protecting against egg contamination (20).

To assay the impact of *sefD* in the hen, egg production was measured following infection of birds with wild-type strain 22079, Δ*sefD* mutant 29108, and complemented mutant 100126. In addition, a wild-type strain (strain 110714) transformed with the complementing plasmid that lacked the *sefD* insert (wild-type transformant) was also assayed. Blood calcium was measured, because microcracks have been observed to increase significantly following infection of chickens with *S.* Enteritidis (16, 25). Blood calcium and egg production are functions of the host that are tightly regulated, involving a delicate balance of dietary factors, vitamin D, parathyroid hormone, calcitonin, and estrogen (30); thus, a significant change in either parameter is a sensitive indicator of a major impact on host physiology. Results herein provide insight into the function of a gene within a fimbrial operon that has somewhat resisted characterization within other models of infection.

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TABLE 1 Bacterial strains used for mutagenic characterization of *sefD* in *S. enterica* serovar Enteritidis

Accession no.	Genotype	Description	Source
22079	Parent strain	*S. enterica* serovar Enteritidis PT4, associated with outbreak in poultry	H. Kinde, Davis, CA
27004	Carries pCP20	*Escherichia coli* K-12 derivative	Salmonella Genetic Stock Centre, Calgary, Canada
27005	Carries pKD46	*E. coli* K-12 derivative	Salmonella Genetic Stock Centre, Calgary, Canada
28006	Carries pKD4	*E. coli* K-12 derivative	R. Maier, Athens, GA
28000	Carries pKD46	22079 carrying pKD46	This study
28029	*sefD*::*kan*	28000 with *sefD*::*kan*, pKD46 removed	This study
29108	Δ*sefD*	28029 with the kanamycin cassette removed	This study
100126	pCR2.1-TOPO with *F* (*lacZa′-sefD*$^+$)	29108 complemented with functional *sefD* inserted in pCR2.1-TOPO	This study

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. Table 1 lists the bacterial strains and plasmids used in this study. Phage type 4 (PT4) *Salmonella* Enteritidis 22079 was the wild type (14). It was obtained from a farm environment in California during an index outbreak linked to the introduction of PT4 *S.* Enteritidis into chicken flocks in the United States (17). Total DNA and RNA were isolated from 10 ml of brain hear infusion (BHI) broth inoculated with single colonies grown at 37°C or 42°C for 16 h or other times, as indicated. Plasmids were obtained from 10 ml Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter). Medium was supplemented with 50 μg/ml kanamycin or 200 μg/ml ampicillin, as appropriate.

Isolation of DNA. Total DNA was extracted from approximately 10^9 bacterial cells (1 ml of culture) using a Qiagen DNeasy tissue kit following the protocol for bacteria (Qiagen, Valencia, CA). Plasmids were isolated from 1 ml culture with a Qiagen Spin QIAprep miniprep kit. Plasmid DNA was eluted in 50 μl EB (10 mM Tris-HCl [pH 8.5]). The concentration and purity of DNA samples were measured using a NanoDrop 1000 (Wilmington, DE) spectrophotometer at λ values of 230, 260, and 280 nm. The optical density at 260 nm/280 nm was greater than 1.70 before further processing.

PCR. Primers used for PCRs are listed in Table 2. Oligonucleotide primers were designed using PrimerQuest software (Integrated DNA Technologies), and all PCRs followed recommended parameters. Primers for generating the site-specific knockout, namely sefD_KO_F/R, were designed to contain 40 bp homology to *sefD* at the 5′ end of the primer and 20 bp homology to the kanamycin resistance cassette at the 3′ end. The PCR mixtures included 100 nM each primer and a 1× concentration of Applied Biosystems (Carlsbad, CA) GeneAmp Fast PCR master mix in a final reaction volume of 20 μl. Reactions were done in an Applied Biosystems Veriti 96-well fast thermal cycler system. PCR products were visualized on Invitrogen precast 2% agarose E-Gel48 gels (Carlsbad, CA) under UV light of 302-nm wavelength. Amplicons were obtained using a Qiagen QIAquick gel extraction kit.

Mutagenesis of *sefD*. Mutagenesis was performed using the bacteriophage λ Red system (8). *Salmonella* Enteritidis PT4 wild-type strain 22079 was made electrocompetent as previously described (24). Plasmid pKD46 was electroporated into *S.* Enteritidis 22079 competent cells using a Bio-Red Gene Pulser X cell electroporation system (2,500 kV, 200 Ω, 25 μF; Hercules, CA). Cells carrying pKD46 were selected by plating on LB agar supplemented with 100 μg/ml ampicillin overnight at 30°C. Confirmed transformants were frozen in 20% glycerol at −80°C. Confirmed mutants for *sefD*::*kan* were assigned accession number 28029. The kanamycin cassette can create polar effects on downstream genes in the same operon (18). This issue is addressed further in text that follows.

Complementation. Mutant 29108 was complemented by inserting the respective gene into plasmid pCR2.1-TOPO (20). Successful transformants were selected under high antibiotic selection pressure (200 μg/ml ampicillin) to result in high numbers of plasmid copies with the cells. The orientation of the gene was confirmed by sequencing (Retrogen, Inc., San Diego, CA) using universal M13 sequencing primers. Strains confirmed to be complemented for Δ*sefD* were frozen in 20% glycerol and given accession number 100126. To measure the impact of the plasmid alone on the biology of wild type, *S.* Enteritidis strain 22079 was transformed following the procedure outlined in the TOPO TA cloning kit with the pCR2.1-TOPO vector. The resulting strain, strain 110714, was wild-type 22079 with the pCR2.1 plasmid containing the control PCR product insert. It is referred to here as the wild-type transformant.

Assessing stability of complementing plasmid. Plasmid stability was assessed by PCR for cultures of strains 100126 and 110714 that were passaged 5 times from brilliant green (BG) agar (Accumedia) to BHI broth (Difco BD, Franklin Lakes, NJ). Passage was done at 37 and 42°C for 16 h in the absence of antibiotic (data not shown). To check for the presence of *sefD*, PCR was with forward (F) and reverse (R) primer pairs *sefD* or pCR2.1 complement (sefD_c1 and sefD_c2 primers or sefD_comp primers) (Table 2). To check for plasmid, PCR was performed with primer pCR2.1 (Table 2). Plasmid-containing strains from the 5th passages were stored for use.

Determination of relative transcription levels of *sefD*. The relative levels of transcription of *sefD* by experimental strains were determined by quantitative real-time quantitative reverse transcription-PCR (qRT-PCR) of cDNA using the housekeeping gene *gyrB* (SEN3652) for normalization of data. Total RNA was isolated from 1 ml culture using a commercial kit (Ambion RiboPure kit; Applied Biosystems). After processing of cells according to directions, sample was transferred to a spin column. The final volume of 50 μl was treated with DNase, pelleted, and transferred to a fresh tube. RNA was converted to cDNA as directed (high-capacity RNA-to-cDNA kit; Applied Biosystems). Master mix was prepared in PCR tubes and contained 2× reverse transcription buffer, 20× reverse transcriptase enzyme mix, and nuclease-free H_2O. RNA concentrations were determined by nanodrop assay and varied from 25 to 70 ng/μl. RNA was added to get 100 ng/reaction mixture in volumes ranging from 5 to 25 μl/reaction mixture. PCR was done at 37°C for 60 min and 95°C for 5 min, and then the reaction mixture was held at 4°C. qRT-PCR was conducted using an Applied Biosystems 7500 real-time PCR system. Optical reaction PCR plates (Applied Biosystems) were loaded with TaqMan gene expression assay mixture using 1 μl *sefD* and *gyrB* mixture of reporter probes and primers (20×) (Table 2), 2× TaqMan gene expression master mix, and nuclease-free H_2O.

Confirmation of *sefC* transcription in *sefD* mutant. In *S.* Enteritidis, *sefC* overlaps *sefD*. To confirm that construction of the *sefD* mutant did not alter *sefC* expression, total RNA samples were converted to cDNA as described above for cultures of wild type, mutant, and complemented mutant. PCR was done at 37°C for 60 min and 95°C for 5 min, and then the mixture was held at 4°C using F/R primers sefD_c1 and sefC_c1 specific for *sefD* and *sefC*, respectively (Table 2). PCR amplicons were electrophoresed and visualized as described above. Samples for confirmation

TABLE 2 Primers and probes used in this research

Primer	Orientation	Primer sequence (5′ to 3′)	Amplicon size (bp)
sefD_KO_F	Forward	TGAATCAGTATAATTCGTCAATACCTAAGTTCATTGTCTCGTGT AGGCTGGAGCTGCTTC	374
sefD_KO-R	Reverse	TATGCTTATTAAATATGTGTCAACAGGAATGTCTCCATTCCA TATGAATATCCTCCTTAG	374
sefD_c1_F	Forward	TGAATCAGTATAATTCGTCAATACCTA	422
sefD_c1_F	Reverse	TATGCTTATTAAATATGTGTCAACAGG	422
sefD_c2-F	Forward	GTGCAAATGAATCAGTATAATTCGTC	110 mutant, 453 parent
sefD_c2-R	Reverse	TTATAATTCAATTTCTGTCGCATATATGC	110 mutant, 453 parent
sefC_c1_F5	Forward	CTTGGTGTTGGGGTTGGGGTT	1,099
sefC_c1_R5	Reverse	GGCCCAGAACTTTTGCCCCA	1,099
sefD_comp_F	Forward	GTGCAAATGAATCAGTATAATTCGTC	453
sefD_comp_R	Reverse	TTATAATTCAATTTCTGTCGCATATATGC	453
sefD_qpcr_F	Forward	GGGCATATATTATTAGAGGTCAGAAT	65
sefD_qpcr_R	Reverse	GTTGCCAGTCTTCTCCACCTATT	65
sefD reporter (FAM[b])	NA[a]	ACTCAGCCCATAAGCTT	65
gyrB_qpcr_F	Forward	GTGAAATGACCCGTCGTAAAGG	75
gyrB_qpcr_R	Reverse	GCAGTCCGCCAGTTTGC	75
gyrB reporter (FAM)	NA	CAGGCCCGCTAAATC	75
pCR2.1_F	Forward	ATGTGGCGCGGTATTATCCCGTAT	1,002
pCR2.1_R	Reverse	ACAGAGTTCTTGAAGTGGTGGCCT	1,002

[a] NA, not applicable.
[b] FAM, 6-carboxyfluorescein.

of *sefC* were obtained at 37 and 42°C at 6 time points every 2 h starting 10 h after incubation.

PM. Wild-type strain 22079, Δ*sefD* strain 29108, complemented mutant 100126, and wild-type transformant 110714 were analyzed by phenotype microarray (PM; Biolog; Hayward, CA) (2). PM was performed at 42°C for 48 h, and plates were sealed with plastic covers. PM using plates PM01 to PM10 was repeated at least twice for each strain. Only runs where the A1 negative-control wells of PM plates 1 to 4 were negative were included for evaluation. The height of the growth curve (based on respiratory activity units) was used as the comparison parameter between strains. Data were not zeroed for comparisons. A peak-height difference of greater than 50 respiratory units (RU) but less than 100 RU was considered significant if it repeated. If it did not repeat, it was considered a marginal difference not useful for distinguishing phenotype. A change in global phenotype was defined as observation of a significant difference in more than 10% of the 920 wells present in plates PM01 to PM10.

Infection of hens. Specific-pathogen-free Leghorn hens between 30 and 45 weeks of age were housed singly in layer cages as 4 treatment groups. Each room housed a minimum of 12 hens. Three of the treatment groups were challenged with bacteria suspended in 0.5 ml of phosphate-buffered saline (PBS; 10 mM phosphate, 138 mM NaCl, 2.7 mM KCl [pH 7.4]; Sigma-Aldrich) by intramuscular (i.m.) injection. The hens in the fourth room were injected i.m. with 0.5 ml of PBS. Hens received on average of $(17 \pm 6.1) \times 10^6$ CFU in trial 1, $(18 \pm 3.4) \times 10^5$ CFU in trial 2, and $(21 \pm 7.4) \times 10^6$ CFU in trial 3, determined by serial dilution. Egg production was recorded for 7 days before and 21 days after infection. For experiments 1 and 2, hens were euthanized at 26 days postinfection. Spleens, cecal contents, and intestinal contents were collected. In the third experiment, 3 to 4 hens were sacrificed weekly for 3 weeks in order to recover spleens. Procedures with hens, including methods for humane euthanasia, were approved by the facility Institutional Animal Care and Use Committee (OPH OLAW assurance number A4298-01).

Assay for blood calcium. Blood was collected every 2 to 3 days for 21 days from the alar vein of three birds per group starting on the day of infection. Blood was clotted at ambient temperature for 1 h. Cells were pelleted in a Sorvall Legend RT centrifuge at 3,000 rpm for 15 min. Serum was transferred and stored at 4°C. Plasma samples were submitted for assay (Athens Δy Diagnostic Labs, GA). A total of 9 samples per group were collected for 2 experiments.

Culture of *S.* Enteritidis from spleen and cecal and intestinal contents. Methods for isolation of *Salmonella* Enteritidis from spleen and cecal and intestinal contents were previously described (13, 20). Colonies were confirmed to be *Salmonella* species using an Enterotube II system (Difco BD) and further processed for genotype confirmation by PCR using the appropriate primers listed in Table 2.

Statistical analysis. The Student *t* test was used to examine if differences between treatment groups were statistically significant. Levels of significance were as follows. Probability values (P) of >0.05 were not considered significantly different. P values of ≥ 0.02 and ≤ 0.05 were considered possibly significant. P values of <0.02 were considered highly significant. Error bars showing standard deviation (SDs) are indicated on graphs and were derived from three separate experiments.

RESULTS

The wild type had significantly lower expression of *sefD* than the complemented mutant. (Table 3) shows results from transcriptional analysis of strains by qRT-PCR measured from 3 different colonies per day on 3 different days. Wild-type strain 22079 and complemented mutant 100126 grown at 37°C had average $\log_{10}$ transcriptional fold differences of 2.09 ± 0.128 and 5.94 ± 0.168, respectively, compared to the mutant. At 42°C, the respective averages were 1.07 ± 0.138 and 4.25 ± 0.113. Thus, transcription of *sefD* was significantly lower for the wild type than for the complemented mutant ($P < 0.01$), and the higher growth temperature significantly decreased transcription for both strains ($P < 0.01$). Transcription of *sefD* by 3 other field isolates of *S.* Enteritidis grown at 37°C was 2.39 ± 0.247, which was not significantly different from the relative transcription observed for wild-type 22079 ($P = 0.07$). These results suggest that transcription of *sefD* is commonly observable in *S.* Enteritidis but that it is significantly suppressed at 42°C.

To explore conditions that favored transcription of *sefD*, the three test strains (wild-type strain 22079, Δ*sefD* mutant 29108, and complemented mutant 100126) were grown at 28, 37, and

TABLE 3 Relative quantification of *sefD* transcription by *S.* Enteritidis after normalization to *gyrB* using the comparative threshold cycle method ($\Delta\Delta C_T$)

Sample[a]	Temp (°C)	C_T[b]		ΔC_T	$\Delta\Delta C_T$	Fold difference (range)	Log_{10} fold difference
		Target	Endogenous reference				
29108_01	37	34.75 ± 0.30	23.20 ± 0.02	11.54 ± 0.30	0.00 ± 0.30	1.00 (0.81–1.23)	
29108_02	37	34.08 ± 0.34	22.73 ± 0.17	11.34 ± 0.38	−0.20 ± 0.38	1.15 (0.88–1.50)	0.06
29108_03	37	35.11 ± 0.11	22.86 ± 0.15	12.25 ± 0.19	0.70 ± 0.19	0.61 (0.54–0.70)	−0.21
22079_01	37	27.66 ± 0.51	23.39 ± 0.33	4.28 ± 0.61	−7.27 ± 0.61	154.06 (100.86–235.31)	2.19
22079_02	37	27.47 ± 0.31	22.99 ± 0.14	4.48 ± 0.34	−7.07 ± 0.34	133.90 (105.74–169.56)	2.13
22079_03	37	28.28 ± 0.24	23.18 ± 0.08	5.09 ± 0.25	−6.45 ± 0.25	87.43 (73.39–104.15)	1.94
100713_01	37	15.28 ± 0.16	23.08 ± 0.09	−7.80 + 0.19	−19.34 ± 0.19	663,314.41 (583,432.91–754,132.98)	5.82
100713_02	37	14.64 ± 0.04	23.47 ± 0.32	−8.83 ± 0.32	−20.37 ± 0.32	1,358,264.80 (1,089,504.79–1,693,322.77)	6.13
100713_03	37	15.12 ± 0.14	23.07 ± 0.11	−7.95 ± 0.17	−19.49 ± 0.17	736,333.60 (652,378.57–831,092.84)	5.87
29108_01	42	33.05 ± 0.08	22.15 ± 0.12	10.90 ± 0.15	0.00 ± 0.15	1.00 (0.90–1.11)	
29108_02	42	33.52 ± 0.16	22.16 ± 0.06	11.37 ± 0.17	0.46 ± 0.17	0.72 (0.64–0.82)	−0.14
29108_03	42	33.12 ± 0.10	21.67 ± 0.07	11.45 ± 0.12	0.55 ± 0.12	0.68 (0.63–0.74)	−0.17
22079_01	42	30.26 ± 0.03	22.41 ± 0.00	7.85 ± 0.03	−3.05 ± 0.03	8.30 (8.12–8.48)	0.92
22079_02	42	30.05 ± 0.18	23.11 ± 0.13	6.94 ± 0.22	−3.96 ± 0.22	15.59 (13.42–18.11)	1.19
22079_03	42	29.96 ± 0.27	22.66 ± 0.08	7.30 ± 0.28	−3.60 ± 0.28	12.13 (9.96–14.78)	1.08
100713_01	42	18.41 ± 0.39	21.49 ± 0.27	−3.08 + 0.47	−13.98 ± 0.47	16,208.54 (11,686.20–22,480.94)	4.21
100713_02	42	18.58 ± 0.32	22.21 ± 0.22	−3.63 ± 0.39	−14.53 ± 0.39	23,609.28 (18,003.49–30,960.56)	4.37
100713_03	42	19.46 ± 0.35	22.36 ± 0.21	−2.90 ± 0.41	−13.81 ± 0.41	14,324.53 (10,792.55–19,012.38)	4.16

[a] Three colonies per strain were analyzed, and each sample was run in triplicate. Approximately 200 ng RNA was used per sample, except for 42°C samples from strains 22079_01, 22079_02, and 29108_01, where ~160 ng was used.
[b] C_T, threshold cycle.

42°C. Relative levels of mRNA were analyzed by RT-PCR at 24-h intervals. The expression of *sefD* was prolonged up to 120 h when complemented mutant 100126 was grown at 28°C (Fig. 1). Transcription was suppressed for the complemented mutant over time as the temperature increased to 42°C (Fig. 1). It was reported that transcription of *sefD* can be tightly regulated and that it is more likely to be present during late exponential phase than stationary phase (11). Further analysis of the three strains at an earlier time point of 10 h showed that *sefD* was expressed by wild type when grown at 42°C for 10 h but not at 16 h (data not shown). These results suggest that *sefD* transcription is more quickly suppressed at host body temperatures by the wild type than it is by the complemented mutant. Results suggest that the wild type appears to the host as a functional *sefD* mutant, whereas the complemented mutant retains transcription after infection. Controls facilitating analysis of data were successful recovery of mRNA for *sefC* in all strains, which is the gene immediately upstream of *sefD* (data not shown), and qRT- and RT-PCR of *gyrB*, which is a housekeeping gene used as an endogenous reference (Table 3).

Complementation of *sefD* resulted in a global decrease in metabolism. Very few metabolic differences were found when Δ*sefD* mutant 29108 was compared to wild-type 22079 and to wild-type transformant 110714 by phenotype microarray analysis. Of the 920 metabolic conditions included in the array, 53 (5.76%) gave variable results between trials using a peak height of 50 RU as a cutoff for the background signal. The number was reduced to 17 of 920 (1.85%) metabolites at a peak-height cutoff at 100. However, the 17 metabolites in question varied between trials of the same strain. Thus, none of the metabolites that showed variation between the mutant, wild type, and wild-type transformant were reliable for denoting phenotypic differences. These results indicated that the three strains had substantially similar metabolic profiles, despite finding some sporadic variation between trials.

Comparison of the Δ*sefD* mutant to its complement showed a global difference in metabolism. Using a peak-height cutoff of 100 RU, 342 of 920 (37.17%) metabolites differentiated the complemented mutant from the mutant, wild type, and wild-type transformant (Table 4). In addition, 318 of the metabolites in the set

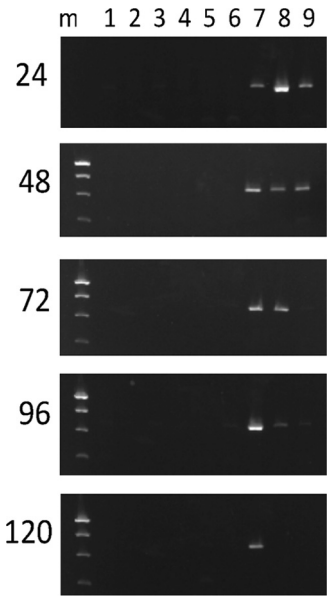

FIG 1 Transcription of *sefD* during stationary phase. Hours of growth are shown in the left column. Lane m, DNA ladder showing bands at 2,000, 800, 400, and 200 bp (top to bottom); lanes 1 to 3, wild-type strain 22079 grown at 28, 37, and 42°C, respectively; lanes 4 to 6, mutant 29108 at the same respective temperatures; lanes 7 to 9, complemented mutant 100126 at the same respective temperatures.

TABLE 4 Student's *t* test analysis (tail 1, type 2) of egg production

Analysis type and strain or group	*t*-test *P* value	
	Parent	Complemented mutant
Variation in egg production between treatment groups		
Mutant	0.294[a]	
Complemented mutant	0.019[c]	
Uninfected hens	0.015[c]	0.295[a]
SDs between treatment groups		
Mutant	0.151[a]	
Complemented mutant	0.035[b]	
Uninfected hens	0.114[a]	0.264[a]

[a] Results are not significantly different ($P \geq 0.05$).
[b] Results are possibly significantly different ($P > 0.02$ and < 0.05).
[c] Results are highly significantly different ($P \leq 0.02$).

reliably repeated between trials; thus, most of the observed differences within a single trial were reliable indicators of distinctive phenotypes. Complementation of *sefD* resulted in a pervasive loss of metabolic capabilities and growth. The class of metabolite with the most differences between strains was N source, which accounted for 131 of the 318 (41.2%) metabolites that varied repeatedly between mutant and complemented mutant (Fig. 2).

Absence of *sefD* increased virulence of *S.* Enteritidis in hens. Daily egg production decreased significantly for hens infected i.m. with *S.* Enteritidis wild-type 22079 and Δ*sefD* mutant 29108 (Fig. 3). The Δ*sefD* mutant produced the most severe reduction in egg production postinfection, with *P* values of 0.006 and 0.005 in comparison to egg production for hens infected with complemented mutant and/or uninfected controls, respectively (Table 4). Infection of hens with the Δ*sefD* mutant produced a significant increase ($P = 0.004$) in the SD of daily egg production in comparison to that for hens infected with the complemented mutant (Table 4), whereas infection with wild type did not ($P = 0.035$). Egg production of hens challenged with complemented mutant 100126 did not differ significantly from that for controls (Fig. 3).

Changes in blood calcium levels (mg/dl) following infection had the same pattern observed for egg production. Hens infected with wild-type 22079 and Δ*sefD* mutant 29108 had significant drops in blood calcium compared to the control birds (Table 5). In contrast, the blood calcium level of hens infected with complemented mutant 100126 was not significantly different from that of control birds after infection (Table 5).

Absence of *sefD* was associated with persistence of *S.* Enteritidis in spleens. *S.* Enteritidis was recovered from 23 of 34 (67.6%) spleens from hens challenged with wild-type strain 22079, 20 of 31 (70%) spleens from hens infected with Δ*sefD* mutant 29108, 9 of 35 (25.7%) spleens from hens challenged with complemented mutant 100126, and none of the spleens from control hens. No *Salmonella* isolates were found in intestinal contents. One cecal sample was positive for wild type.

It appeared that the complemented mutant could lose the transforming plasmid at some point after infection, despite its having undergone selection for stable transformation at the body temperature of the hen. For example, 4 of 13 (30.8%) and 0 of 12 spleens were culture positive for the complemented mutant. To investigate what was happening in more detail, 3 to 4 hens were sacrificed weekly for 3 weeks in the third experiment. Any culture-positive samples were evaluated by PCR and visualization of the band of the expected size. Isolates recovered from hens challenged with wild-type strain 22079 (positive for *sefD*, negative for transforming plasmid) and Δ*sefD* mutant 100126 (negative for *sefD*, negative for plasmid) had the expected genotype in all cases. No positive spleens were isolated in the first week after hens were infected with complemented mutant 100126. At the end of week 2, 1 of 3 (33.3%) spleens was positive and the one isolate had lost the plasmid. At 3 weeks postinfection, all 4 spleens (100%) that were culture positive had lost the complementing plasmid. Loss of the plasmid after initial infection did not appear to alter egg production, because no significant differences that were not a result of the initial infectious dose were seen between trials. These results suggest that the complemented mutant reverted to mutant status at some point postinfection but that reversion did not alter the kinetics of egg production following the initial infection. Further analysis is needed to explore how the absence of *sefD* contributes to persistence in the spleens of hens.

Absence of *sefD* may be associated with higher mortality in hens. The percentages of average mortality over 3 experiments for wild-type strain 22079, Δ*sefD* mutant 29018, and complemented mutant 100126 were, respectively, 5, 13, and 0. These results suggest that mortality was higher for hens infected with the mutant. The clinical impression of the hens, as observed by two veterinarians, was that those infected with the mutant were sicker overall than those infected with wild type in regard to suppression of appetite and lethargy. Additional experimentation is needed to confirm if the Δ*sefD* mutant was more virulent than wild type in a manner that could impact epidemiology on farm. It appears that low-level expression of *sefD* imparted some protection to the hen against the worst effects of infection with *S.* Enteritidis.

DISCUSSION

The role of *sefD* in the virulence of *S.* Enteritidis has been difficult to determine. Transcriptional analysis for the most part agrees with previous analyses by others, because *sefD* was detected in wild type at a similar time point during growth at late log phase

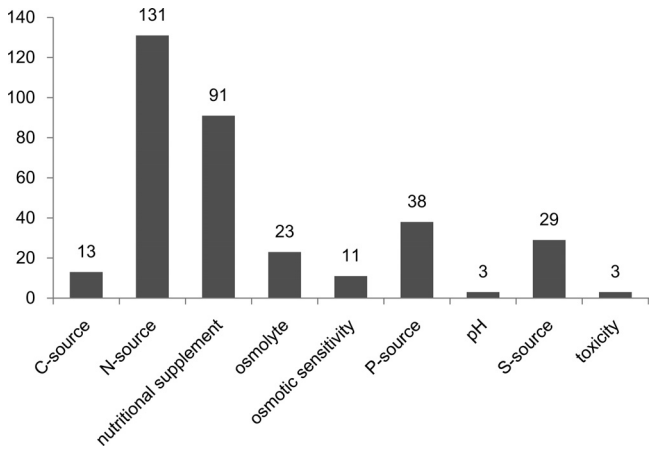

FIG 2 Classes of compounds that vary between the *S.* Enteritidis Δ*sefD* mutant and its complement that produce transcripts at 42°C. A total of 342 metabolic conditions had peak heights that were more than 100 RU different between strains. The number of metabolites in each class of metabolite or growth condition is shown.

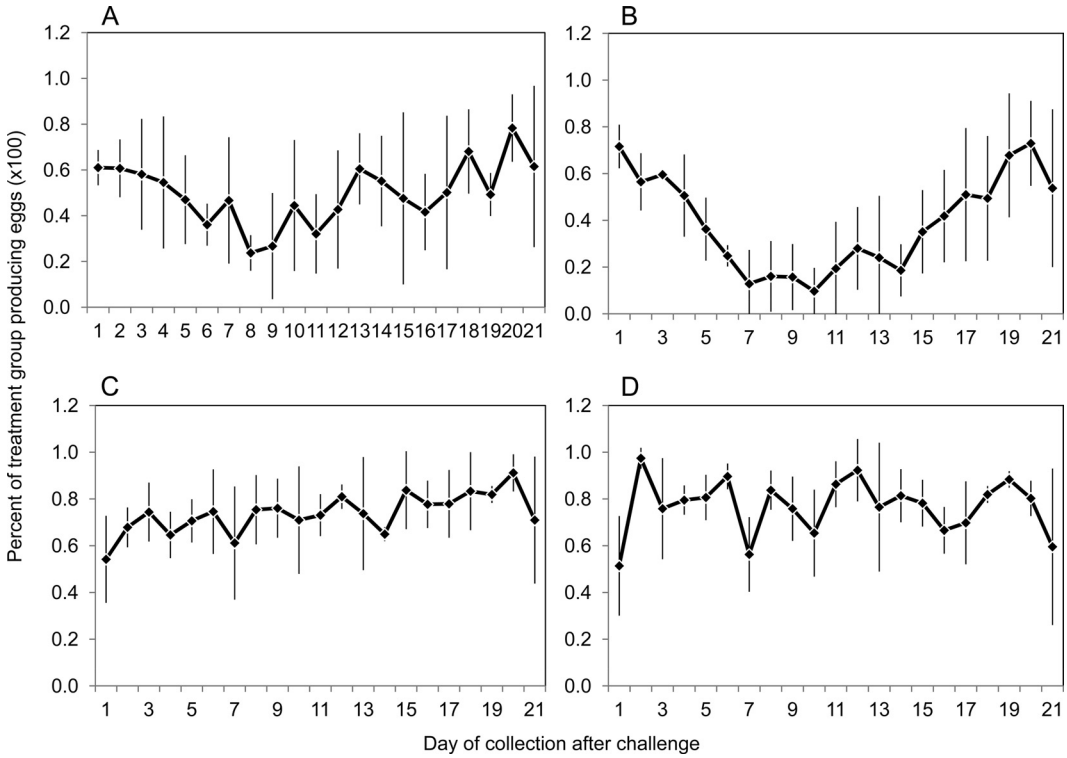

FIG 3 Egg production by hens infected with *S.* Enteritidis that varies in *sefD*. *x* axis, day of egg collection postinfection; *y* axis, percent daily egg production (number of eggs collected daily/number of hens). (A) Wild-type (22079)-infected hens; (B) mutant (29108)-infected hens; (C) complemented mutant (100126)-infected hens; (D) uninfected hens. Data are from 3 separate trials. See Table 5 for analysis of significance between groups. Hen ages were 30, 44, and 46 weeks at the start of the experiment. The route of infection was intramuscular, and doses were delivered in a total volume of 0.5 ml PBS. Infectious dosages were (17 ± 6.1) × 10^6 CFU, (21 ± 7.4) × 10^6 CFU, and (18 ± 3.4) × 10^5 CFU, respectively, for the three ages of hens.

(11, 29). However, wild type produced less *sefD* at the higher body temperature of the hen. Thus, wild-type strains appear to be similar to the Δ*sefD* mutant in regard to virulence attributes in the hen because transcription was suppressed. Given that other field isolates appeared to have similar levels of transcription, *S.* Enteritidis appears to be a functional *sefD* mutant in the hen, despite having an intact gene. Transcription of *sefD* may occur within a few microenvironments in the hen, and a requirement for specific cofactors would complicate analysis. Further research is needed to identify cofactors, environmental conditions, and regulatory elements that impact expression of *sefD* at different temperatures.

***sefD* mitigates symptoms in the hen and may contribute to environmental adaptation.** Constitutive expression of *sefD* was associated with mitigation of illness in hens, as assayed by measuring blood calcium and egg production. It appears that even a trace of *sefD* mitigates disease, because birds infected with the wild type had less mortality than those infected with the mutant. Infection of hens with the mutant produced wide standard deviations in experimental parameters, which supports the clinical impression that some individuals were especially affected. Low-level expression of *sefD* by *S.* Enteritidis may facilitate the infection pathway that results in egg contamination by moderating the host response, which would make it difficult for farmers to recognize that hens were infected.

TABLE 5 Blood calcium for treatment groups[a]

Strain or group	Avg blood calcium (mg/dl) ± SD	
	30-wk-old hens	46-wk-old hens
Parent (22079)	17.0 ± 5.04[b]	21.5 ± 7.71[b]
Mutant (29108)	20.2 ± 7.13[b]	19.1 ± 7.75[b]
Complemented mutant (100126)	26.8 ± 3.30[c]	28.6 ± 3.02[c]
Uninfected hens	26.1 ± 2.82	29.5 ± 1.38

[a] Samples were collected from 3 birds every 2nd or 3rd day for 21 days postinfection (9 samples per group) for use in two experiments.
[b] Highly significant difference compared to uninfected hens.
[c] No significant difference compared to uninfected hens.

The fleeting nature of *sefD* transcription informs future research. Transcription of *sefD* was evident at the ambient temperature of 28°C in stationary phase, but it was suppressed at the mammalian body temperature of 37°C and strongly suppressed at the avian body temperature of 42°C. This result supports the finding of Ogunniyi et al. (29) that SEF14 may help bind *S.* Enteritidis to inanimate objects. Since *sefD* is required to form the SEF14 fimbria, it is possible that it is present on the cell surface the moment that a hen encounters *S.* Enteritidis in the environment. Perhaps SEF14 facilitates adherence to the oral cavity following ingestion from feed but is then suppressed to allow the initially infecting cells to colonize further into the gastrointestinal tract. We suggest that SEF14 may be a fimbria that facilitates transition between the environment and host (3, 12). Conceptualizing SEF14 as a fleeting contributor to the infection pathway resulting in egg contamination facilitates the design of further experimen-

tation. For example, it will be important to investigate if SEF14 contributes to successful immunization of hens. The alternative hypothesis is that the SEF14 fimbria has no role at all in the infection pathway resulting in egg contamination and instead matters within an unexplored environment. Further research is needed to investigate if SEF14 contributes to colonization of other vectors, such as insects.

***sefD* transcriptional levels may contribute to the variation in response to infection between individuals.** Monitoring of blood calcium was useful for assessing the severity of illness in the individual host animal infected with *S.* Enteritidis. It is possible that there are fundamental differences between the mouse and hen models of infection (13). Topics of interest for further research would be to evaluate if the chicken regulates calcium homeostasis differently than the rodent and if macrophages from the chicken give the same results as those from the mouse. Further research is needed to see if the large standard deviations associated with infection of hens by *S.* Enteritidis Δ*sefD* help to predict severity of illness within a flock.

The gene *sefD* may be a coordinating element for virulence attributes. It is not evident why a putative outer membrane fimbria-associated protein would impact metabolism. For example, phenotype microarray analysis strongly indicated that loss of *sefD* was linked to the ability of more pathogenic strains to grow on a wider range of metabolites. A concept to consider is that *sefD* is a gene that coordinates virulence attributes, namely, loss of a fimbrial structure in coordination with the emergence of metabolic properties that are important for successful colonization and infection of the host.

Genomic databases may be skewed toward reporting the absence of *sefD* in *Salmonella enterica*. Nearly all reported whole-genome databases for *Salmonella enterica* lack *sefD* (15). It is known that both *S.* Enteritidis and *S.* Pullorum vary in having complete ORFs for *sefD* (31). *S.* Gallinarum, which produces high mortality in chickens, lacks *sefD*. What is not understood is why three genetically related but phenotypically diverse serotypes that all have the ability to contaminate eggs generate variation in this one operon at the same place. Is predilection of *Salmonella enterica* toward mutation of *sefD* a subtle genetic event that imparts a fitness advantage to the pathogen in the host? Could the SNP in *sefD* be an allelic variation that has become more prevalent because the gene is less important now than it used to be for transmission of *Salmonella* under modern methods of production? It may be prudent to explore if *sefD* defines a type of phase variation and if its loss contributes to the persistence of *Salmonella enterica* in the chicken and to virulence in mammalian hosts. In summary, it is important to note that *sefD* is only one of many genes that vary in content in *S.* Enteritidis (15). The impact that *sefD* has on bacterial phenotype is thus further obfuscated by the combinatorial complexity that defines *Salmonella* Enteritidis as a pathogen. These results provide some insight into how to proceed with the characterization of *sefD* in the chicken.

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