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RfaH Promotes the Ability of the Avian Pathogenic *Escherichia coli* O2 Strain E058 To Cause Avian Colibacillosis

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Avian pathogenic *Escherichia coli* (APEC) infection causes avian colibacillosis, which refers to any localized or systemic infection, such as acute fatal septicemia or subacute pericarditis and airsacculitis. The RfaH transcriptional regulator in *E. coli* is known to regulate a number of phenotypic traits. The direct effect of RfaH on the virulence of APEC has not been investigated yet. Our results showed that the inactivation of *rfaH* significantly decreased the virulence of APEC E058. The attenuation was assessed by *in vivo* and *in vitro* assays, including chicken infection assays, an ingestion and intracellular survival assay, and a bactericidal assay with serum complement. The virulence phenotype was restored to resemble that of the wild type by complementation of the *rfaH* gene in *trans*. The results of the quantitative real-time reverse transcription-PCR (qRT-PCR) analysis and animal system infection experiments indicated that the deletion of *rfaH* correlated with decreased virulence of the APEC E058 strain.

Isolates of extraintestinal pathogenic *Escherichia coli* (ExPEC) cause infection in nearly every organ and anatomical site in humans and animals. Among ExPEC strains, avian pathogenic *E. coli* (APEC) strains are responsible for serious extraintestinal diseases of poultry, causing high morbidity and mortality in chickens and turkeys, leading to great economic losses (1, 2). APEC infection causes a variety of severe infections, including acute fatal septicemia, subacute pericarditis, and airsacculitis. Most often, APEC strains infect chickens, turkeys, ducks, and other avian species through fecal dust via the respiratory tract. APEC strains possess genes coding for various virulence factors for colonizing and invading the host, including adhesins, toxins, polysaccharide coatings, protectins, invasins, and iron acquisition systems (3, 4). Epidemiological studies have shown that APEC isolates predominantly belong to the O1, O2, and O78 serogroups (5, 6).

From attachment and colonization to the host cells to systemic invasion, bacteria sense the environment and regulate the expression of virulence genes that are required for effective pathogenesis. A complex regulatory network exists in *E. coli* that mediates this response to environmental signals (7, 8). In *E. coli* and many other bacterial species, a regulatory protein, RfaH, acts as a transcriptional antiterminator that reduces the polarity of long operons encoding cell components (9, 10). RfaH was first discovered as a regulator of lipopolysaccharide (LPS) synthesis in *Salmonella enterica* (11) and *E. coli* (12). Later, RfaH was shown to be essential for the expression of other cell components encoded on long operons in *E. coli*, including the expression of F plasmid (13), different capsules (14–16), and hemin uptake receptor (17), as well as the toxins alpha-hemolysin (18, 19) and cytotoxic necrotizing factor 1 (20). RfaH-dependent operons share a short *cis*-acting element termed *ops* (for operon polarity suppressor) that is essential to allow RfaH to function (21). How all these virulence factors evolved to utilize the same core regulatory mechanism still awaits discovery.

Previous research showed that disruption of the *rfaH* gene in uropathogenic *E. coli* strain 536 results in a significant decrease in virulence (22). As *rfaH* seems to be conserved among various bacteria, its role in the regulation of virulence of other ExPEC pathogens has been suggested. The purpose of this investigation was to assess the hypothesis that *rfaH* is critical to the virulence of APEC E058. To that end, an isogenic *rfaH* mutant of APEC O2 strain E058 was constructed using lambda Red recombination as described previously (23). The mutant was tested for its contribution to APEC E058 pathogenicity, including *in vivo* and *in vitro* assays to reveal the pathogenic traits.

MATERIALS AND METHODS

Bacterial strains, primers, and growth conditions. The strains and plasmids used in this study are listed in Table 1, and the primers are listed in Table 2. Bacteria were routinely cultured in Luria Bertani (LB) broth at 37°C with aeration. Antibiotics were added at the following concentrations: chloramphenicol (Cam), 30 μg/ml, and ampicillin (Amp), 60 μg/ml.

Construction of *rfaH* deletion mutant. Deletion of *rfaH* from the chromosome of APEC E058 was performed using gene replacement methods based on the lambda Red recombinase system (23). E058 was initially electroporated with pKD46 to express Red recombinase. The E058Δ*rfaH* strain was constructed as follows: the *rfaH* gene (GenBank accession number M94889.1) was amplified by PCR using the primers HF and HR (Table 2). The products were cloned into the pMD18-T simple vector to form pMD-*rfaH*. To insert the chloramphenicol acetyltransferase (*cat*) cassette into *rfaH*, reverse PCR was adopted. The reverse PCR product was amplified from pMD-*rfaH* using the primers SHF and SHR (Table 2). The *cat* cassette was obtained from pkD3 using primers CF and CR (Table 2). The *cat* cassette was then inserted into the *rfaH* genes at the EcoRV site.

Reverse transcription-PCR analysis. To determine whether the insertion had a polar effect on the upstream or downstream genes, total RNA was extracted from log-phase bacteria of strains E058 and E058Δ*rfaH* using the RNAiso Plus kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. Contaminating DNA was removed

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
Strains		
E058	Wild-type avian *E. coli* serotype O2	41
E058Δ*rfaH*	E058Δ*rfaH*::*cat*	This study
ReE058Δ*rfaH*	complementation of E058Δ*rfaH*	This study
DH5α	λ^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	Invitrogen
Plasmids		
pMD18-T Vector	TA cloning vector; Amp	TaKaRa
pGEM-T Easy Vector	TA cloning vector; Amp	Promega
pMD-*rfaH*	*rfaH* cloned into pMD 18-T vector	This study
pMD-*rfaH-cat*	Cat-resistant gene inserted into pMD-*rfaH*	This study
pT-P_{native}*rfaH*	pGEM-T Easy carrying *rfaH* ORF and its putative native promoter	This study
pKD46	Amp; expresses λ Red recombinase	23
pKD3	*cat* gene, template plasmid	23

from the samples, and cDNA synthesis was performed using the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa) according to the manufacturer's instructions. Primer sets for PCR amplification of the target genes *tatD* (TF/TR), *rfaH* (HF/HR), and *ubiD* (UF/UR) in cDNA samples are listed in Table 2. In parallel, PCRs were performed with genomic DNA as a positive control and RNA samples without activation of reverse transcription (RT) as a negative control. The PCR products were resolved on 0.8% agarose gels and visualized by ethidium bromide staining.

Complementation of the *rfaH* mutant. To complement the deleted *rfaH*, the entire *rfaH* gene, including its natural putative promoter, was amplified and subcloned into the pGEM-T Easy vector using primer pairs RHF and RHR (Table 2), forming pT-P_{native}*rfaH*, and the resulting plasmid was transformed into *E. coli* DH5α. Positive colonies were selected and identified by PCR and sequencing, and the purified recombinant plasmid pT-P_{native}*rfaH* was transformed to the mutant strain E058Δ*rfaH*.

Bactericidal assay with SPF chicken serum. Complement-sufficient specific-pathogen-free (SPF) chicken serum was prepared and pooled from 10 SPF chickens. A bactericidal assay was performed in a 96-well plate as described previously but with the following modifications (24).

TABLE 2 Primers designed and used in this study

Primer	Primer sequence (5′–3′)	Target gene, plasmid, or region
Targets of primers for gene mutagenesis		
HF	CTCGGATCCTTAGAGTTTGCGGAACTC (BamHI site underlined)	*rfaH*
HR	CTCAAGCTTTTACTGTACTGCAAGCGC (HindIII site underlined)	*rfaH*
SHF	CTCGATATCGCCGTATCCTGGTGATAA (EcoRV site underlined)	pMD-*rfaH*
SHR	CTCGATATCGTGGTATGAATCACTTCC (EcoRV site underlined)	pMD-*rfaH*
CF	CTCGATATCTTGTGTAGGCTGGAGCTGCT (EcoRV site underlined)	pKD3
CR	CTCGATATCATGGGAATTAGCCATGGTCC (EcoRV site underlined)	pKD3
RHF	CTGCGGCAGTTTATCAAACAG	Upstream region of *rfaH*
RHR	GAAGCGATTATTGAGCTGGCC	Downstream region of *rfaH*
TF	ATGGGCTACTCATCACCG	*tatD*
TR	CACCAGGCAGTTTATCCAG	*tatD*
UF	CGAAAACCCTAAAGGCTACT	*ubiD*
UR	CCACTCCTGATAATCCAACG	*ubiD*
Targets of primers for real-time PCR		
rfaH RT-F	TCGGGCTCGGTGAAA ATG	*rfaH*
rfaH RT-R	ATCGACGCCGTATCCTGGTG	*rfaH*
chuA RT-F	TACCGACCCAACCAACAG	*chuA*
chuA RT-R	GTCCCGAACGCCAAAAT	*chuA*
kpsM RT-F	TGCCAGACATCTCGTTCC	*kpsM*
kpsM RT-R	TCGCTTCAATAGCACCAAT	*kpsM*
traT RT-F	CAGCAATCAAGAAGCGTAAC	*traT*
traT RT-R	TTCGCCTGAATCCAGTAGTA	*traT*
iutA RT-F	CGATGCCACCTTGCTTGA	*iutA*
iutA RT-R	CAGCCCGTTACTGACGAATG	*iutA*
iucD RT-F	AACAACCTTATTTACCACCCTG	*iucD*
iucD RT-R	TCTGTCCTCCACCAACCAC	*iucD*
iroN RT-F	GGCGATACGCAAAACAGT	*iroN*
iroN RT-R	CCCAGTCCCAGATACCATT	*iroN*
iss RT-F	AACACCAAAGGAAACCATCA	*iss*
iss RT-R	CCGAGCAATCCATTTACGA	*iss*
tsh RT-F	GGCGCTTACTTTGATGTGAT	*tsh*
tsh RT-R	GCTGTTACGACGCATTGAGA	*tsh*
vat RT-F	GCTTTGGTTCGCTCGTGTT	*vat*
vat RT-R	ATCTGCCTGGATGGTTGTGTT	*vat*
cvaC RT-F	AATGTCTCCATCCGGTTTA	*cvaC*
cvaC RT-R	TCTTCCCGCAGCATAGTT	*cvaC*
astA RT-F	AAA AGTCGGCTGGTGGAA	*astA*
astA RT-R	CTGCGTGGCATTTGAGGA	*astA*

SPF chicken serum was diluted to 5, 12.5, 25, 50, and 100% in pH 7.2 phosphate-buffered saline (PBS). Bacteria (10 μl containing 10^6 CFU) were inoculated into reaction wells containing 190 μl of the diluted SPF chicken serum, 100% heat-inactivated SPF chicken serum, or PBS alone and then incubated at 37°C for 30 min. Serial dilutions (1:10) of each well were plated onto LB agar plates. The resulting colonies were counted after 24 h of incubation.

Virulence assay *in vivo*. To assess attenuation of the *rfaH* mutant, groups of 10 1-day-old chickens were infected with the wild-type strain and its isogenic *rfaH* mutant, as well as the complementation strain. The birds were treated in the experiments in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals of the People's Republic of China (Approved by the State Council on 31 October 1988). Strains were grown to exponential phase, collected, washed twice in PBS, and then adjusted to the appropriate doses. Birds were inoculated via the air sac with 0.1 ml of each bacterial suspension containing 10^7 CFU bacteria. The number of bacteria contained in the inoculum was confirmed by plating on LB agar. Negative controls were injected with PBS. Mortality was monitored until 7 days postinfection (p.i.).

Colonization and persistence of the mutant. Animals were infected with the wild-type strain, the mutant, and the complementation strain to determine their colonization and persistence abilities during systemic infection. Briefly, 5-week-old SPF chickens (white leghorn; Jinan SPAFAS poultry Co. Ltd., Jinan, China) were inoculated via the left thoracic air sac with 10^8 CFU of each bacterial suspension. After 24 h, 15 chickens from each group were euthanized and examined for macroscopic and histological lesions. The hearts, livers, spleens, lungs, and kidneys of the birds were collected, weighed, and triturated. The numbers of bacteria were determined by plating serial dilutions of the homogenates onto LB agar plates.

Coinfection assays. For *in vivo* competitive-coinfection assays, 5-week-old white leghorn SPF chickens were inoculated with cultures of the wild-type strain, E058, and its mutant, E058Δ*rfaH*, mixed at a ratio of 1:1 (1×10^8 CFU for each strain) via the left air sac. The chickens were provided with food and water *ad libitum*; 24 h after infection, the hearts, livers, spleens, lungs, and kidneys of the inoculated chickens were collected, weighed, and homogenized, and serial dilutions were plated on LB medium with or without chloramphenicol for selection of mutant or total bacteria, respectively.

Ingestion and intracellular-survival assay. Ingestion and intracellular-survival tests were performed principally as described previously (25). The avian macrophage cell line HD-11 was cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco, New York, NY) with 10% fetal bovine serum (FBS) (PAA, Pasching, Australia) in 24-well cell culture plates. The cells were maintained at 37°C in a 5% CO_2 environment, and the plates contained $\sim 2 \times 10^5$ cells per well. The plates were incubated for 24 h prior to the ingestion assay. Bacteria were added to the cells at a multiplicity of infection (MOI) of 100 for 1 h to allow ingestion. The wells were washed with PBS, and the appropriate cell culture medium containing 100 μg/ml gentamicin was added for 1.5 h to kill extracellular bacteria. At this time (T_0) and at additional, different incubation periods (6, 12, and 24 h), cells were washed using PBS and lysed with 1 ml 0.1% Triton X-100 for 5 min at room temperature. Released bacteria were diluted and plated for viable counts. The ingestion ratio was determined by dividing the number of ingested bacterial cells at T_0 by the number of bacteria in the initial inoculation. Intracellular growth was expressed as the change (*n*-fold) in the bacterial number at an additional incubation time point relative to the initial number of ingested bacteria (T_0).

RNA isolation and qRT-PCR. To determine whether the cell culture medium or interaction with the host can induce expression of the transcriptional regulator RfaH, quantitative RT (qRT)-PCR was applied to analyze the *rfaH* expression of E058 under both *in vitro* and *in vivo* conditions. For *in vitro* measurements, E058 was cultured with aeration to the logarithmic phase in DMEM with 10% fetal bovine serum or in LB medium. Cultures were pelleted (10 min; 2,500 × *g*; 4°C) and stored at −70°C for RNA isolation. For *in vivo* samples, 1-day-old chickens (n = 10) were infected with 10^8 CFU of E058 by the air sac route. Five hours following challenge with E058, 9 ml blood was collected with 1 ml anticoagulant (0.5% sodium citrate). The anticoagulant and blood were centrifuged (5 min; 500 × *g*; 4°C), and the supernatants containing bacteria were collected. The supernatants were centrifuged (10 min; 2,500 × *g*; 4°C), and the bacterial pellet was frozen at −70°C until RNA extraction. RNA isolation and cDNA synthesis were performed using the RNAiso Plus kit and the PrimeScript RT reagent kit with gDNA Eraser according to the manufacturer's instructions (TaKaRa). qRT-PCR was performed to determine the *rfaH* transcription level of E058 under both *in vitro* and *in vivo* conditions using SYBR premix *Ex Taq* (TaKaRa) and *rfaH* RT-F/R primers (Table 2). Data were normalized to the housekeeping gene *gapA* transcript.

To analyze how gene expression was affected by the loss of RfaH, we performed qRT-PCR analyses for various virulence genes in APEC. Overnight cultures of strains were diluted 1:100 in fresh LB until the logarithmic phase of growth. Following RNA isolation and cDNA synthesis, qRT-PCR was performed to determine the transcription levels of the virulence genes using SYBR premix *Ex Taq* and gene-specific primers (Table 2), and the data were normalized to the *gapA* transcript.

Statistical analysis. Differences between groups were analyzed using the Statistical Package for the Social Sciences (SPSS version 15.0; SPSS, Chicago, IL). P values of less than 0.05 were considered to be significant.

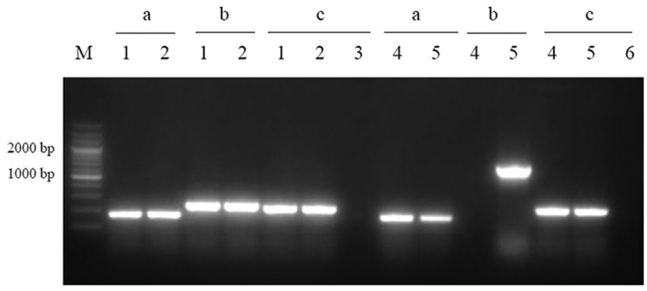

FIG 1 Detection of *tatD*, *rfaH*, and *ubiD* gene expression in E058 and E058Δ*rfaH* by RT-PCR. The RT-PCRs were performed using the following templates: cDNA derived from total RNA of E058 (lanes 1) and E058Δ*rfaH* (lanes 4) and genomic DNA from E058 (lanes 2) and E058Δ*rfaH* (lanes 5). Reaction sets contained the following primers: for lanes labeled "a," TF/TR; for lanes labeled "b," HF/HR; and for lanes labeled "c," UF/UR. The negative controls (lanes 3 and 6) used total RNA as the template without activation of RT. A 200-bp marker (TaKaRa) was used as the molecular size standard (M).

RESULTS

Deletion of *rfaH* does not affect the growth kinetics of APEC E058. An *rfaH* deletion of E058 was created using gene replacement methods based on the lambda Red recombinase system. Part (126 bp) of the *rfaH* gene (489 bp) was deleted, and the *cat* cassette was inserted. To determine whether the insertion had a polar effect on the upstream or downstream genes, total RNA samples extracted from the parental E058 and E058Δ*rfaH* were analyzed by RT-PCR using primer sets designed for *tatD*, *rfaH*, and *ubiD* (Table 2). Compared to the parental E058, the insertion of the *cat* gene in E058Δ*rfaH* only disrupted the transcription of the *rfaH* gene but had no influence on the expression of the genes upstream and downstream of *rfaH* (Fig. 1).

For complementation, the open reading frame (ORF) and natural putative promoter of *rfaH* were amplified from E058 and cloned into the pGEM-T Easy vector. The resulting recombinant plasmid, pT-P_{native}*rfaH*, was transformed into the mutant strain E058Δ*rfaH*, yielding the complementation strain, ReE058Δ*rfaH*.

TABLE 3 Death rates elicited by APEC E058 and its isogenic *rfaH* mutant after air sac infection

Strain	No. dead/total no. (%) after air sac infection of birds (1×10^7 CFU)
E058	10/10 (100)
E058Δ*rfaH*	0/10 (0)
ReE058Δ*rfaH*	10/10 (100)

Compared with the parental strain, the colonies of the isogenic E058Δ*rfaH* mutant on the LB agar plates were similar to those of the parental strain, and the growth rate in LB broth in logarithmic phase was also similar to that of the parental strain (data not shown).

The *rfaH* deletion attenuates APEC E058 in birds. APEC E058 is a virulent avian pathogenic strain that could cause typical avian colibacillosis, with bacteria invading air sacs, blood, pericardial fluid, and the typical fibrinous lesions. To investigate whether RfaH plays a role in the virulence of the strain, groups of 10 1-day-old birds were infected with 1×10^7 CFU of the wild-type strain and its isogenic *rfaH* mutant, as well as with the complementation strain, and the mortality of the birds was observed for 7 days after the challenge; the mortality rates were 100%, 0%, and 100%, respectively (Table 3). Loss of *rfaH* resulted in abolishment of virulence of E058 in birds, while *trans*-complementation of the mutant strain with *rfaH* completely restored its virulence. The results provide evidence that RfaH is an important virulence factor in the pathogenesis of APEC infection.

RfaH protein is required for serum resistance of APEC E058. Resistance to serum has been associated with *E. coli* causing infections in poultry and extraintestinal infections in other species. Our results showed that loss of the regulatory protein RfaH results in high susceptibility to chicken serum in strain E058Δ*rfaH* (Fig. 2).

Mutation in *rfaH* reduces the survival ability of APEC E058 within chicken macrophages. Analysis of the capability of the *rfaH* mutant to be ingested by avian macrophages was carried out using an ingestion assay in the avian macrophage HD-11 cell line. The *rfaH* mutant showed a highly increased ingestion ratio of macrophages compared to the wild-type strain or the complementation strain (Fig. 3).

To determine whether the loss of RfaH has an influence on intracellular survival, the yield of wild-type strain E058 was compared to that of its *rfaH* mutant recovered following an additional 6, 12, and 24 h of incubation. At 6, 12, and 24 h p.i. (beginning at T_0), the numbers of wild-type bacteria recovered from macrophages were 1.47%, 1.75%, and 2.45% of the inoculum, which were 4-, 5-, and 7-fold higher than those of the primary ingested bacteria (T_0) (Fig. 4), respectively. In contrast, the *rfaH* mutant showed impaired growth in macrophages, with numbers representing 2.15%, 0.36%, and 0.11% of the inoculum at 6, 12, and 24 h p.i., which were 2-fold higher at 6 h p.i. and 3- and 10-fold lower at 12 and 24 h p.i. than the primary ingested bacteria (T_0), respectively (Fig. 4). *trans*-complementation of the *rfaH* mutation restored the ability of the *rfaH* mutant to grow in macrophages at wild-type-strain levels, with numbers representing 1.05%, 1.44%, and 1.95% of the inoculum at 6, 12, and 24 h p.i., respectively. The *rfaH* mutant exhibited significantly reduced intracellular yields relative to its parental wild-type or complementation strain at 12 and 24 h p.i. ($P < 0.01$).

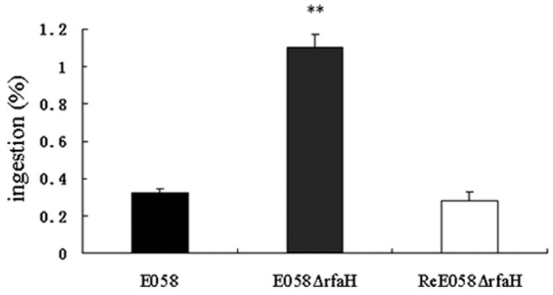

FIG 3 Ingestion of wild-type strain E058, isogenic mutant E058Δ*rfaH*, and complementation strain ReE058Δ*rfaH* by HD-11 cells. The values represent the average data from three independent experiments. The error bars indicate standard deviations. **, statistically significant differences between the wild-type and mutant strains ($P < 0.01$).

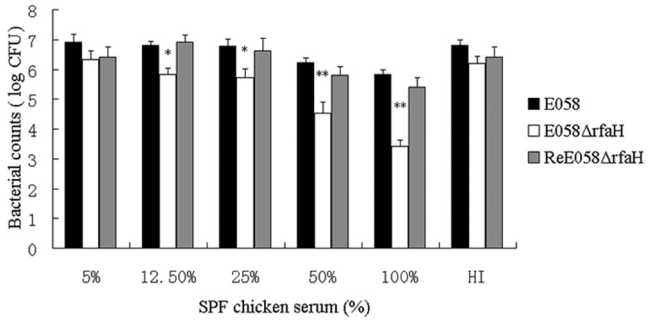

FIG 2 Bactericidal activities of SPF chicken serum against wild-type strain E058 and an isogenic mutant. HI represents 100% of heat-inactivated SPF chicken serum. The data represent an average of three trials. The error bars indicate standard deviations. The asterisks indicate statistically significant differences (*, $P < 0.05$; **, $P < 0.01$).

Effect of *rfaH* during systemic infection *in vivo*. To investigate the effect of the *rfaH* mutation on bacterial pathogenesis, we carried out a colonization and persistence assay or coinfection challenge in a chicken model. To evaluate virulence, the bacterial loads in various tissues were determined. In the colonization and persistence assay, 5-week-old chickens were inoculated via the air

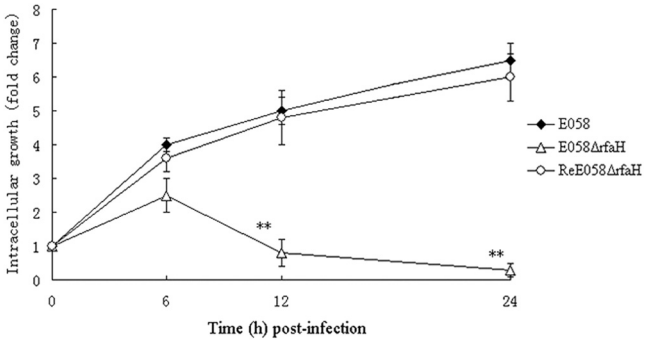

FIG 4 Intracellular growth of bacteria in HD-11 chicken macrophages. The intracellular growth rates of wild-type strain E058, the isogenic mutant E058Δ*rfaH*, and the complementation strain ReE058Δ*rfaH* were compared over a 24-h period. Intracellular bacterial growth is shown as the change in the number of intracellular bacteria following an additional 6, 12, and 24 h of incubation compared to the primary ingested bacteria (T_0). Standard errors of the mean (SEM) for three independent experiments are shown. The asterisks indicate statistically significant differences (**, $P < 0.01$).

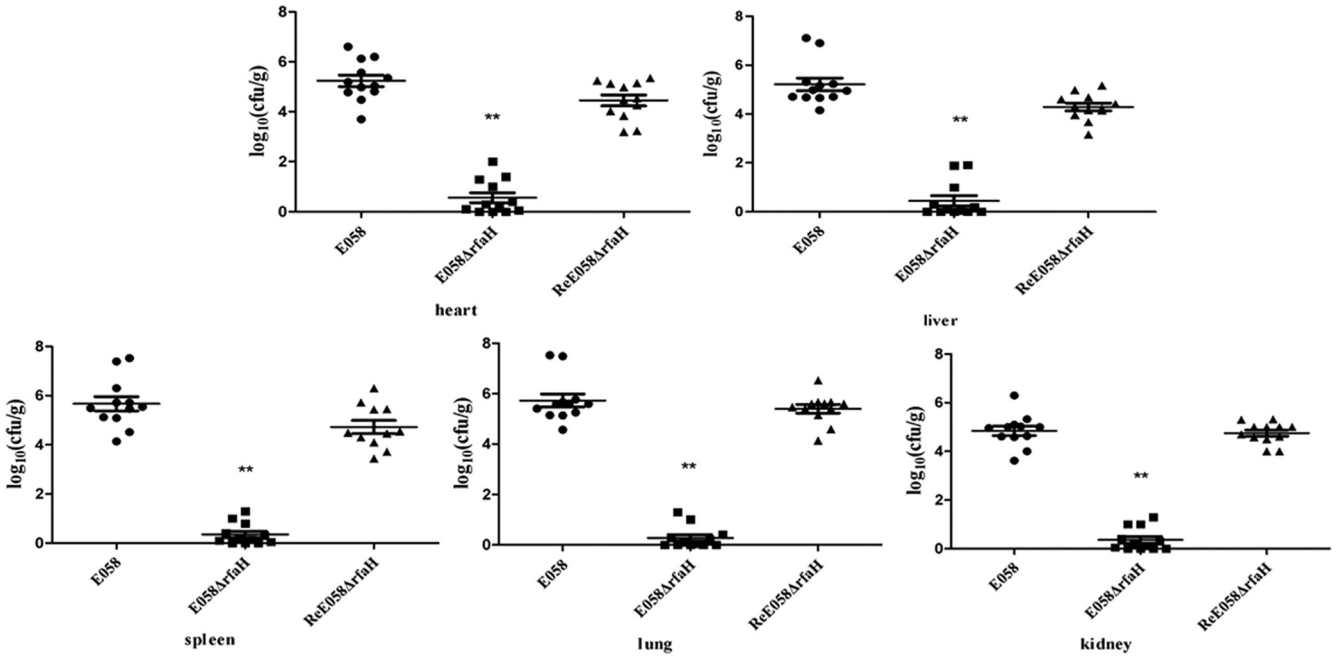

FIG 5 Colonization and persistence of the wild-type strain E058 (●), E058Δ*rfaH* (■), and ReE058Δ*rfaH* (▲) during systemic infection. The data are presented as $\log_{10}$ CFU · g of tissues^{-1}. The horizontal bars indicate the mean $\log_{10}$ CFU · g^{-1}. Each data point represents a sample from an individual chicken. Statistically significant differences as determined by the Mann-Whitney test are indicated by asterisks (**, $P < 0.01$). The error bars indicate standard deviations.

sac with the wild-type strain, its isogenic mutant, and the complementation strain. From the inoculation site, the virulent strain is typically able to invade and infect deeper tissues, generates gross lesions, and causes a systemic infection. However, in this model, the attenuated strain was impaired in its capacity to colonize deeper tissues. Mutants in *rfaH* exhibited significantly reduced colonization compared to the wild type in the organs tested ($P < 0.01$) (Fig. 5). Conversely, the recovered complementation strain in all organs tested was restored to a level similar to that of the wild-type ($P > 0.05$) (Fig. 5).

The competitive-coinfection model demonstrated that E058Δ*rfaH* was outcompeted by the wild-type strain. As shown in Fig. 6, the *rfaH* mutant strain was recovered at significantly lower levels than wild-type E058 in all tested organs ($P < 0.01$). These results indicate that the role of RfaH is critical to allow APEC strain E058 to compete for colonization *in vivo*.

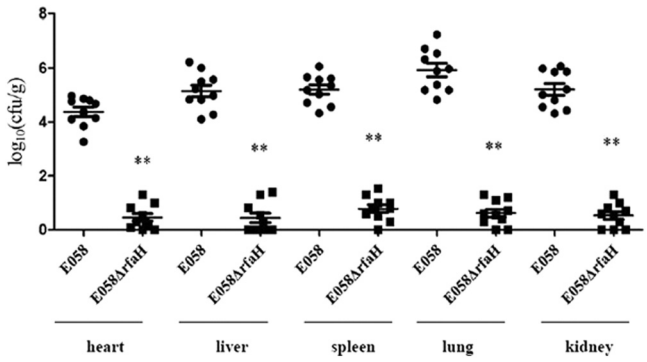

FIG 6 *In vivo* competition assays. E058 (●) and mutant E058Δ*rfaH* (■) were inoculated simultaneously. The data are presented as $\log_{10}$ CFU · g of tissues^{-1}. The horizontal bars indicate the mean $\log_{10}$ CFU · g^{-1}. Each data point represents a sample from an individual chicken. Statistically significant differences in values between E058 and its mutant are indicated by asterisks (**, $P < 0.01$). The error bars indicate standard deviations.

Expression profile of related virulence genes. The results of the cell assays showed a difference in survival ability between the wild-type and mutant strain, which suggests that the cell culture medium or interaction with host cells can induce expression of RfaH. However, our results showed that the expression of *rfaH* in E058 *in vivo* or following culture in the cell culture medium *in vitro* was unaltered compared to that in E058 cultured in LB medium ($P > 0.05$) (data not shown).

Since RfaH has been described as a factor that positively affects gene expression, the transcription profile of virulence genes associated with capsule, iron uptake, serum resistance, autotransporter, colicin V, and the F pilus were analyzed upon *rfaH* inactivation by qRT-PCR *in vitro*. The transcription levels of the *kpsM*, *chuA*, *iucD*, *iutA*, *iroN*, *iss*, *vat*, *tsh*, *cvaC*, *astA*, and *traT* genes were quantified by qRT-PCR. As shown in Fig. 7, the transcription levels of the virulence genes *chuA*, *traT*, *kpsM*, *iss*, and *cvaC* were significantly decreased in the *rfaH* mutant by 0.06, 0.02, 0.05, 0.49, and 0.56 times, respectively ($P < 0.01$), while the other associated virulence genes were not influenced compared to the wild-type strain ($P > 0.05$). The expression levels of these virulence genes were restored in the complementation strain (Fig. 7).

DISCUSSION

APEC is a subset of extraintestinal pathogenic *E. coli* and shares virulence traits with strains isolated from human cases of neonatal meningitis, urinary tract infections, and septicemia. Thus, APEC strains represent a high risk of zoonotic infection (26), and their virulence gene pool may contribute to the emergence of other ExPEC strains (27). APEC O2 strain E058 is a highly virulent iso-

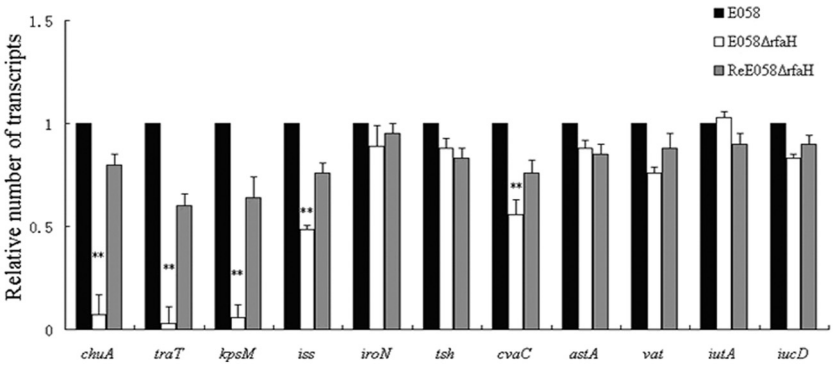

FIG 7 Quantitative RT-PCR analysis of associated virulence gene transcription levels in strains E058, E058Δ*rfaH*, and ReE058Δ*rfaH*. RNA was isolated from strains cultured in LB. Transcript levels were measured in cDNA preparations from each strain and normalized to the *gapA* level, and the results are shown as fold changes relative to the wild-type level. The asterisks indicate statistically significant differences (**, $P < 0.01$). The error bars indicate standard deviations.

late involved in the development of colisepticemia, cellulitis, and respiratory disease, and it belongs to one of the most prevalent serogroups that cause avian colibacillosis.

Bacterial pathogens use specific or global regulators to mediate adaptive responses to the different environments and stresses encountered within the host (28). The infection process requires rapid adaptation to the host environment by alteration of gene expression. RfaH is a transcriptional antiterminator that reduces the polarity of long operons encoding secreted and surface-associated cell components (LPS, capsules, exotoxins, hemin uptake receptor ChuA, and F pilus) involved in the virulence of *E. coli* pathogens.

Since all the structures influenced by RfaH are potential virulence factors, we proposed that mutation of the *rfaH* gene results in a decrease in APEC virulence. Indeed, in a chicken infection model, the virulence of wild-type strain E058 was almost completely abolished through the loss of the *rfaH* gene. The *rfaH* mutant did not exhibit a growth defect, as determined by the generation time in LB at 37°C, indicating that the attenuation observed was not due to a general growth defect. Our results indicated that RfaH regulon activity is largely responsible for APEC attenuation. This statement is based on the fact that the *rfaH* mutant was affected in both *in vivo* and *in vitro* assays.

Our results demonstrated that the resistance to serum is impaired through the inactivation of *rfaH*. This is significant, since there is a correlation between resistance to the bactericidal effects of serum and the capacity of APEC strains to cause septicemia and mortality (29, 30). Serum resistance has been a virulence parameter for APEC. Many phenotype traits contribute to *E. coli*'s serum resistance, including lipopolysaccharide, type 1 fimbriae, capsule, O antigen, and outer membrane proteins (29, 31–33), including OmpA, TraT, and Iss (34–36). APEC strains more often contain ColV plasmids that encode serum resistance (37). We have shown that loss of the regulatory protein RfaH results in high susceptibility to chicken serum in strain E058Δ*rfaH*, which might be explained by decreased expression of *traT* and *iss* (Fig. 7) due to inactivation of the *rfaH* gene.

The most common form of APEC infection in poultry is characterized by initial respiratory tract colonization, followed by a systemic spread to other parts of the body. Avian air sacs do not have cellular defense mechanisms and depend initially on the influx of heterophils, followed by macrophages as a cellular defense (38, 39). The ability to persist within macrophages provides a survival advantage to APEC strains by abrogating elimination by the host immune responses. Moreover, the pathogenic APEC strains are more resistant to killing by chicken macrophages *in vitro* than the less pathogenic strains are (40). Therefore, using the HD-11 chicken macrophage line, we examined the effects of mutation of *rfaH* on bacterial survival within cultured macrophages. The *rfaH* mutant bacteria appeared to be more susceptible to engulfment by the macrophages, while the wild-type or complemented bacteria appeared to resist engulfment, as shown in the ingestion assay (Fig. 3). We have shown that the absence of RfaH results in decreased intracellular growth of APEC in macrophage cells (Fig. 4). As intracellular growth provides a survival advantage to APEC, the phenotype may contribute to virulence attenuation of *rfaH* mutants.

By using an avian experimental model, we demonstrated that inactivation of the *rfaH* gene of E058 leads to attenuation of virulence, since the *rfaH* mutant showed significantly decreased colonization compared with the wild-type strain in all organs tested in the single-strain challenge model (Fig. 5), implying that RfaH plays a critical role in the virulence of APEC E058 in chickens. The E058Δ*rfaH* complementation strain restored virulence and colonized the internal organs of inoculated birds to the same extent as the wild-type strain (Fig. 5). Chickens that were challenged with wild-type strain E058 developed severe bilateral airsacculitis, severe hypertrophy and congestion of the spleen, and moderate perihepatitis. In contrast, the mutant E058Δ*rfaH* caused no conspicuous changes in the tissues mentioned above. The coinfection model provides more sensitivity to differences in the colonization or virulence of the *rfaH* mutant and those of the wild-type strain without involving individual differences of the host. Our results provide evidence that, in contrast to the wild-type strain, the *rfaH* mutant was significantly outcompeted in the chickens (Fig. 6). These results indicate that the ability to produce RfaH is critical to allow APEC E058 to compete for colonization *in vivo*. Meanwhile, the results of the qRT-PCR analysis demonstrated that the inactivation of *rfaH* decreased the transcription level of virulence genes involved in the capsule, hemin uptake, serum resistance, and colicin V synthesis (Fig. 7), which may contribute to the reduced colonization and proliferation capacities of APEC E058.

Taken together, our results confirm that the RfaH regulon plays a major role in APEC E058 virulence and associated traits.

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