Deletion of the c2515 and c2516 Genes Affects Iron Uptake and Virulence of APEC O1 Strain E516

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Avian pathogenic Escherichia coli (APEC), widely spread among poultry, is well-known to cause colibacillosis in chickens, which results in significant losses in poultry industry. The ability to uptake iron in the extra-intestinal environment is prerequisite for APEC survival. For adaptation to the low-iron environments, the bacteria have evolved multiple iron acquisition systems to ensure optimal iron uptake. However, many components of these iron acquisition pathways are still not clearly known. An in silico analysis of the genome of a septicemic APEC O1 strain E516 identified two putative iron transport genes homologous to the c2515 and c2516 genes from uropathogenic E. coli CFT073. In this study, we constructed the single and double gene deletion mutants, and studied their biological characteristic and pathogenic traits through in vitro and in vivo assays. Reverse transcriptase PCR (RT-PCR) analyses demonstrated that the mutations destroying the reading frame of the target genes abolished their transcription. Deletion of the single or double genes of c2515 and c2516 in APEC E516 weakened its ability to produce siderophore. Consistently, the mutants exhibited growth defect under iron-depleted conditions and the intracellular iron levels in the mutants were decreased in comparison with that of the wild-type (WT). Cell infection assays showed that the iron uptake defective mutants were more easily eliminated by the macrophage. Inactivation of the c2515 and c2516 genes affected bacterial colonization of chicken tissues, as well as the 50% lethal dose levels compared with the WT strain. Moreover, the expression levels of several iron uptake-related genes were significantly decreased in the double-deletion mutant. In total, the c2515 and c2516 may involve in siderophore-mediated iron uptake and participate in the pathogenesis of APEC O1 strain E516.

Keywords: avian pathogenic Escherichia coli, iron transport genes, mutant, iron uptake, virulence

INTRODUCTION

Avian pathogenic Escherichia coli (APEC) causes typical extra-intestinal infections in poultry referred to as colibacillosis, causing severe economic losses and hindering the development of the poultry industry (1). Being the most important bacterial pathogen, APEC is not only affecting variety of bird species, but also threatening the human healthy by sharing multiple virulence factors with human extra-intestinal pathogenic E. coli (ExPEC) (2). Among the numerous serogroups identified, the O1, O2, and O78 are the most common serotypes associated with colibacillosis in chickens (3).
Avian pathogenic *E. coli* possesses a variety of virulence genes that produce adhesins, toxins, invasins, serum resistance and iron acquisition factors (4–8). Under infection conditions, the ability of the bacterial pathogen to competitively uptake the endogenous iron determines the outcome of the infections. Iron is an essential nutrient for bacterial growth and plays an important role in regulating numerous cellular processes, including oxygen transport, electron transfer and enzymatic function (9–11). Therefore, effective iron uptake strategy is very critical for the growth and infection of pathogens. Given the limited iron availability, bacteria have evolved a number of iron uptake, transport and utilization systems. Bacteria usually secrete specific high-affinity siderophores to chelate iron and then uptake these iron-binding siderophores into cells via specific cellular transport systems (12, 13). The TonB-ExbB-ExbD transport system is required for the energy-dependent transport of ferric siderophores across the outer membrane (14). The SitABCD is a periplasmic binding protein-dependent ABC transport system that mediates iron transport (15). Iron, IreA, and IutA are considered to be outer membrane receptor proteins, which involve in periplasmic internalization of ferri-siderophores (16, 17). Though numerous iron acquisition pathways have been found, the functions and roles of many components are not fully understood.

We previously sequenced the genome of three different serotypes APEC strains: E516 (O1), E058 (O2), and E522 (O78), among which the E516 strain possess a higher virulence than the other two strains. By comparing these genomes, we found that these three strains possess the similar iron acquisition systems, including heme, enterobactin, salmochelin, aerobactin, and yersiniabactin. However, we identified two putative iron transport genes homologous to the *c2515* and *c2516* genes from uropathogenic *E. coli* CFT073, that are present in E516 genome but absent in the E058 and E522 genome. The *c2515*, encoding a ATP-binding cassette (ABC) transporter that mediates iron import, is thought to transport the ferric-siderophores and enter into the cytoplasm with the help of *c2516*, which encoding plasma membrane permeases for high-affinity iron uptake. Whether these two genes contribute to iron uptake and virulence of *APEC E516* is unknown. Therefore, in this study, we evaluated the phenotypic characterization of APEC strains containing defined mutations in *c2515* and *c2516*. Our report demonstrate the role they play in iron uptake and virulence of the APEC O1 strain E516.

**MATERIALS AND METHODS**

**Bacterial Strains, Plasmids, and Growth Conditions**

All *E. coli* strains, plasmids, oligonucleotide primers, and cell line used in this study are listed in Tables 1, 2. All primers used in amplification of the genes were obtained from Sangon Co. Ltd. (Shanghai, China). *Escherichia coli* was grown on LB agar plates or broth with appropriate antibiotic supplementation. Antibiotics were added at the following concentrations: chloramphenicol, 30 µg/ml and ampicillin, 60 µg/ml.

**Construction of Mutant and Complementation Strains**

The *c2515* (GI: 1037781) or *c2516* (GI: 1037782) gene was deleted from APEC E516 using gene replacement methods based on the lambda Red recombinase system (18). The chloramphenicol-resistance cassette, flanked by the 5’ and 3’ sequences of the *c2515* or *c2516* gene, was amplified from the pKD3 plasmid using VCAT-F/R and DCAT-F/R primers (Table 2), respectively. The *c2515* or *c2516* single deletion mutant was confirmed by PCR and verified by sequencing.

The *c2515* and *c2516* double-deletion mutant was constructed using the same method as the single mutant strain. The chloramphenicol-resistance cassette, flanked by the 5’ sequences of the *c2516* and 3’ sequences of *c2515* gene, was amplified from the pKD3 plasmid using DCAT-F/VCAT-R primers.

For complementation, the coding sequences of the *c2516-c2515* genes, together with their putative promoter regions, were amplified from strain E516 and cloned independently into pACYC184. The purified recombinant plasmid was transformed into the double-deletion mutant strain to generate the complementation strain.

**RT-PCR**

Total RNA was isolated from log-phase bacteria of E516, Δ*c2515*, Δ*c2516*, and Δ*c2515Δ*c2516*, ReΔ*c2515Δ*c2516* using an RNeasy kit (Qiagen) and treated with an on-column Rnase-Free Dnase set. The first-strand synthesis of cDNA was primed with random primers using a high capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA). Primers set for PCR amplification of target genes *c2516*, *c2515*, and *c2516c2515* in cDNA samples were DF/DR, VF/VR, and DF/VR, respectively (Table 2). In parallel, PCRs were performed with E516 DNA as positive controls and cDNA samples without activation of the reverse transcription as negative controls. The PCR products were resolved on 0.8% agarose gels and visualized by GoodView staining.

**Growth Assay**

Growth of all strains in iron-depleted or supplemented medium was examined as previously described (20). Avian pathogenic *E. coli* E516 and its isogenic mutants were cultured overnight in LB broth. Cultures were washed twice and diluted in 10 ml LB, iron-depleted medium [M9 minimum salts, 0.05 mg/ml thiamine, 20% glucose, 0.02 mg/ml L-Tryptopham, 5 mg/ml Casamino acids, 0.1 mM CaCl2, 2 mM MgSO4 and 200 µM 2,2-dipyridyl (DIP) (Sigma, St. Louis, MO, USA)], iron-depleted medium supplemented with 100 µM FeCl3, and the cell density was estimated by spectrophotometry to achieve an approximate starting concentration (OD600 = 0.05). Bacterial growth was measured at 1-h intervals over 12 h by spectrophotometry (OD600). The experiment was performed in triplicate.
### Siderophore Production

The chrome azurol S (CAS) agar diffusion assay was used for detecting siderophore production (21). CAS agar plate was prepared as follows: first, 60.5 mg CAS [TCI (Shanghai) Development Co., Ltd., Shanghai, China] was dissolved in 50 ml deionized water, and mixed with 10 ml iron III solution (1 mM FeCl₃, 10 mM HCl). Under stirring, this solution was slowly mixed with 72.9 mg hexadecyltrimethyl ammonium bromide (HDTMA) [TCI (Shanghai) Development Co., Ltd., Shanghai, China] was dissolved in 50 ml PBS, and the cells were lysed by ultrasonic crash to release intracellular iron. The samples were centrifugated to exclude the impurities and the supernatant was used for iron concentration determination. Deionized water (0.1 mL), iron standard stock solution (0.1 mL) was diluted according to instructions, and sample (0.1 mL) were individually mixed with a chromogenic agent (0.4 mL), boiled for 5 min, and then centrifuged at 3,000 g for 10 min. The supernatant was subsequently collected. Iron content was estimated by measuring the OD at 520 nm (OD₅₂₀) of the supernatant and a standard product curve (y = ax + b) was created. The following formula was used:

$$\text{Iron concentration (mg/L)} = \left( \frac{A_{520} - b}{a \times f} \right)$$

A₅₂₀: sample OD₅₂₀ − deionized water OD₅₂₀
f: Sample dilution factor

### Iron Uptake-Related Gene Expression Analysis

To analyze whether the disruption of c2515 and c2516 affects the production of the known iron uptake systems, the iron uptake-related genes (entA, fepC, irp2, ireA, fecA, fyuA, iutA, chuA, iroN) were selected for comparing the transcription levels between the WT strain and its isogenic mutants. Total RNA was extracted from the WT, double-deletion mutant and complementation strains cultured in iron-depleted medium. Quantitative real-time PCR (qRT-PCR) was performed to determine the transcription levels of the tested genes using SYBR premix Ex Taq and genespecific primers (Table 2), and the data were normalized to 16s rRNA. The relative gene expression levels were calculated using the 2⁻ΔΔCt method.

### Cells Infection Assays

The intracellular survival of the APEC strains was determined using a gentamicin protection assay with chicken macrophage HD-11 cells (19). The cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco, NY, USA) containing 10% fetal bovine serum (PAA Laboratories, Pasching, Austria), and maintained at 37°C in a 5% CO₂ environment in 24-well cell culture plates, with 2 × 10⁵ cells per well. The cells were infected with the bacteria at a multiplicity of infection (MOI) of 100 for 1 h to allow the uptake of the bacteria. After the 1 h infection period, the cells were washed three times with PBS, and were incubated with 100 µg/ml gentamicin for 1 h to kill any extracellular bacteria. At this time point (T₀), the cells were then washed and reincubated with 10 µg/ml gentamicin to prevent extracellular replication, with...
The HD-11 cells were plated on glass coverslips in 12-well-plates at $2 \times 10^5$ cells per well and infected with bacteria at an MOI of 100 CFU per cell as described above. At 12 h post-infection (p.i.), the cells were washed gently with PBS, fixed with 4% para-formaldehyde in PBS, and permeabilized with 0.1% Triton X-100. After blocking with 5% bovine serum albumin, the bacterial cells were incubated with polyclonal chicken anti-APEC O1 serum (1:500) for 1 h at 37°C, washed twice with PBS, and stained with FITC goat anti-chicken IgG (1:500) (Sigma, St. Louis, MO, USA). F-actin was then stained with phalloidin-Alexa Fluor 568 (Thermo Fisher Scientific), and the nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole; Thermo Fisher Scientific). The surviving bacteria in the HD-11 cells were visualized using a laserscanning confocal microscope (Leica TCS SP8 STED).

**Visualization of Surviving Bacteria Within the Macrophage Cells**

The HD-11 cells were plated on glass coverslips in 12-well-plates at $2 \times 10^5$ cells per well and infected with bacteria at an MOI of 100 CFU per cell as described above. At 12 h post-infection (p.i.), the cells were washed gently with PBS, fixed with 4% para-formaldehyde in PBS, and permeabilized with 0.1% Triton X-100. After blocking with 5% bovine serum albumin, the bacterial cells were incubated with polyclonal chicken anti-APEC O1 serum (1:500) for 1 h at 37°C, washed twice with PBS, and stained with FITC goat anti-chicken IgG (1:500) (Sigma, St. Louis, MO, USA). F-actin was then stained with phalloidin-Alexa Fluor 568 (Thermo Fisher Scientific), and the nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole; Thermo Fisher Scientific). The surviving bacteria in the HD-11 cells were visualized using a laserscanning confocal microscope (Leica TCS SP8 STED).

**LD$_{50}$**

For virulence evaluation, the 50% lethal dose (LD$_{50}$) was determined using 1-day-old chick infection model as described previously (22). The chicks were provided with food and water ad libitum and treated in accordance with the Regulations
The Colonization Ability of the Mutants
We further evaluated the role of these genes in APEC colonization and virulence. Briefly, 3-week-old white leghorn SPF chickens were inoculated into the left thoracic air sac with suspension containing $10^7$ CFU of the WT strain E516 or mutant derivatives. After 24 h of infection, 10 chickens of each group were euthanatized and hearts, livers, spleens, lungs, and kidneys were aseptically collected. Samples were weighed, triturated and homogenized. The number of the bacteria colonized in the organs was determined by plating 10-fold serial dilutions of the homogenates on LB agar plates.
FIGURE 2 | Growth curves of E516 wild-type strain and its mutants. (A) The E516, Δc2515, Δc2516, Δc2515Δc2516, and ReΔc2515Δc2516 strains were grown in LB broth at 37°C, and their growth was determined by measurement of optical density at 600 nm (OD_{600}). (B) Growth curves of the E516 and its mutants in iron-depleted MM9 medium. (C) Growth curves of the E516 and its mutants in iron-depleted MM9 medium supplemented with 100 μM FeCl₃. The data represent averages of three independent assays (*P < 0.05, **P < 0.01).
FIGURE 3 | The iron uptake ability between the WT and the mutants strains. (A) The CAS agar diffusion assay. Strains E516 and ReΔc2515Δc2516 complementation rendered orange halos larger than that of the mutants. (B) The intracellular iron levels of the WT, mutant, and complemented strains. The bacterial strains were cultured in iron-depleted MM9 medium to the log phase. The cellular iron contents of the WT, mutant, and complemented strains were determined using an iron colorimetric assay kit (*P < 0.05, **P < 0.01).
FIGURE 4 | Quantitative real-time reverse transcription-PCR (qRT-PCR) analysis of gene expression. The expression levels of the iron uptake-related genes (entA, fepC, irp-2, ireA, fecA, fyuA, iutA, chuA, iron) in the WT strain, Δc2515Δc2516, and ReΔc2515Δc2516 were tested by qRT-PCR. The relative gene expression levels were calculated using the 2^{ΔΔCt} method (*P < 0.05).

Statistical Analysis
Differences between groups were analyzed using the commercially available statistical software GraphPad Prism v7.0 (GraphPad Software). P < 0.05 was considered statistically significant and P < 0.01 was highly significant.

RESULTS
Genetic Analysis and Construction of the Single- and Double-Deletion Mutants for c2515 and c2516
Based on the sequenced genome of APEC O1 strain 516, primers DF/DR and VF/VR were designed to amplify two putative iron transport genes 0199 and 0200. PCR detection showed that these two genes present in the genome of E516, but absent in the genome of E058 and E522 (Data not shown). The nucleotide homology was assessed by searching the non-redundant nucleotide collection at GenBank. The searches revealed that the 0199 and 0200 genes share 100% identity with the uropathogenic E. coli CFT073 c2515 and c2516 genes, respectively. These two genes are adjacently located, with the c2516 being the upstream gene of c2515 (Figure 1A).

After construction of the mutants by λ Red recombinase-mediated mutagenesis, sequencing analysis confirmed that the Cat cassette had been inserted into the genome at the predicted position. RT-PCR analysis demonstrated that the insertion had disrupted the expression of the target genes (Figure 1B). These mutants were named as E516Δc2515, E516Δc2516, and E516Δc2515Δc2516, respectively.

Growth in Iron-Depleted and Iron-Supplemented Medium
To determine whether these two genes play roles in iron uptake, the growth of strains with either a single deletion of c2515 or c2516, or the double deletion of both genes, was compared. Growth of all strains in iron-depleted and iron-supplemented medium was observed by measurement of OD_{600} every hour for 12 h of cultivation. The results showed that all three mutants grew at rates similar to that of their wild-type strain in LB medium (Figure 2A). However, they manifested significantly retarded growth compared with the WT strain under the iron-depleted culture conditions, while the double-mutant grew more slowly than either single-mutant strain (Figure 2B). The mutant strains’ growth defects in iron-depleted medium were reversed by supplementation with 100 μM FeCl₃ (Figure 2C).

The Siderophore Production and Cellular Iron Uptake
The CAS agar diffusion assay was adopted to detect the siderophore production. The orange halo produced by the bacterial strains on CAS agar is indicative for the siderophore production. After culture, the orange halos of the WT strain E516 and the complementation strain were significantly larger than those of the mutant strains on the CAS plate (Figure 3A),
indicating that the c2515 and c2516 genes may involved in the production of the siderophore.

We tested further, whether the deletion of these two genes would affect the cellular iron uptake. Our results demonstrated that, when mutant cells grew in LB-Fe medium (LB+DIP), they had about 2–7-fold less free intracellular iron than WT cells. Furthermore, the free intracellular iron level is dramatically decreased in the Δc2515Δc2516 mutant as compared with either
To understand the effect of iron uptake on the intracellular survival of APEC within chicken macrophages, we further tested under iron-depleted conditions using qRT-PCR. Our data showed that the expression levels of entA, fepC, and iutA were significantly decreased in the double mutant by 0.61, 0.68, and 0.56 times, respectively ($P < 0.05$), while those of the other iron uptake-associated genes were not significantly changed compared to the wild-type strain. The expression levels of these genes were restored in the complementation strain (Figure 4).

**Transcription Levels of Iron Uptake-Related Genes in the Mutant**

The effects of disruption of $c2515$ and $c2516$ on the expression of the iron uptake-related genes were further tested under iron-depleted conditions using qRT-PCR. Our data showed that the expression levels of entA, fepC, and iutA were significantly decreased in the double mutant by 0.61, 0.68, and 0.56 times, respectively ($P < 0.05$), while those of the other iron uptake-associated genes were not significantly changed compared to the wild-type strain. The expression levels of these genes were restored in the complementation strain (Figure 4).

**Iron Uptake-Defective Mutants Survive Poorly Within Chicken Macrophages**

To understand the effect of iron uptake on the intracellular survival of APEC, we compared the bacterial yields of mutants and the WT strains within the chicken macrophages. Initially, the $\Delta c2515$ or $\Delta c2516$ single and $\Delta c2515\Delta c2516$ double mutants showed significantly decreased internalization compared with the WT strain (Figure 5A). During the following survival period, both the single and double mutants survived poorly within the HD-11 macrophages, as shown by the lower bacterial yield recovered at each specified time point (Figure 5B). The intracellular survival of the complementation strain was higher than that of the single or double mutants strains, but it did not return completely to the wild-type level. These results were further confirmed by immunofluorescence analysis in which HD-11 macrophages displayed higher intracellular bacterial loads of WT or complemented strain than the resulting single or double mutants at 12 h post-infection (Figure 5C). These results showed that knockout of the $c2515$ or $c2516$ genes impaired the intracellular survival of APEC E516 in macrophages.

**Evaluation of the Virulence of Mutants by $LD_{50}$**

According to the $LD_{50}$ results, the WT strain E516 exhibited a high lethality in birds, while the knockout mutants of these two genes reduced the virulence of E516 in varying degrees. A 7-fold reduction of the virulence was observed in the $\Delta c2515$ mutant, whereas the reduction was 5-fold in absence of $c2516$. The double mutant showed a more dramatic decrease in virulence by 10-fold (Table 3), while the complementation of the double mutant restored its virulence.

**The Colonization Ability of the Mutants**

The colonization ability was assessed in WT strain and mutants of $c2515$ and $c2516$ using the chicken septicemia model. At 24 h post-infection, the WT strain E516 colonized more efficiently in the internal tissues, with the bacterial loads in the selected organs ranged from $7.7 \times 10^7$ to $1.1 \times 10^8$ CFU per gram of tissues. Compared with the WT strain E516, the mutant E516$\Delta c2515\Delta c2516$ showed significantly reduced bacterial numbers in all tested organs, with the bacterial loads ranged from $1.5 \times 10^4$ to $4.6 \times 10^5$ CFU per gram of tissues ($P < 0.05$, Figure 6). The $\Delta c2516$ mutant showed a decreased colonization in kidney of challenged chickens than the WT bacteria ($P < 0.05$, Figure 6). The complementation strain Re$\Delta c2515\Delta c2516$ colonized the chicken tissues similarly to the WT strain ($P > 0.05$, Figure 6).

**DISCUSSIONS**

Avian pathogenic *E. coli* utilize a variety of virulence factors to enhance its infection. However, strains of different serogroups or genotypes may possess different virulence potentials. In our previous study, we have identified the O1 strain E516 that are of substantially greater virulence than O2 strain E058 and O78 strain E522. We further explored the genomic difference that might underlie this difference in virulence. By comparing the genomes of these three strains, we identified two putative iron transport genes $c2515$ and $c2516$ that are located on the genomes of E516, but absent in the genomes of E058 and E522. Sequence blast in NCBI database showed that these two genes were also found in several other ExPECs such as uropathogenic *E. coli* UTI89 and the neonatal meningitis *E. coli* O18. Because iron plays significant roles in metabolism, the ability of APEC to sequester iron from their hosts is a major virulence determinant (24). In this study, we sought to evaluate the role of these two genes in iron uptake and virulence of APEC E516.

The iron chelator dipyridyl was used to produce iron-limited conditions that inhibited the growth of APEC strains. Our data suggest that $c2515$ or $c2516$ is necessary for efficient growth of APEC E516 under iron limitation but not under iron sufficiency. Since the chelator may also sequester other metals, addition of extra-iron to the iron-depleted medium for a regain of growth is a more direct way to confirm that the drop in growth is likely to be due to a decrease in ability to sequester iron. Indeed, the optical density of cultures of the mutant strains in iron-depleted medium supplemented with FeCl$_3$ regained levels similar to that of the wild-type strain, suggesting that the mutants strain’s growth defect in MM9 medium where dipyridyl was added was the results of iron depletion. Because...
FIGURE 6 | Colonization of the WT strain E516 (●), Δc2515 (▲), Δc2516 (▼), Δc2515Δc2516 (■) and ReΔc2515Δc2516 (●) during systemic infection. A, Heart; B, Liver; C, Spleen; D, Lung; E, Kidney. Data were presented as log\(_10\) (CFU/g) of tissues. Horizontal bars indicated the mean values. Each data represented a sample from an individual chicken. Statistically significances as determined by the Mann-Whitney-test were indicated by asterisks (* \(P<0.05\); ** \(P<0.01\)).
of the observed growth defect under iron-limited conditions, we analyzed siderophore production in mutant strains lacking one or two genes. Phenotypical analysis revealed a weak CAS halo in the either single- or double-deletion strains, while complementation restored the ability of the double mutant to produce a CAS halo similar to that of WT strain. A defect in siderophore production might result in lowered iron transport and enrichment in the bacterial cells. Indeed, the levels of iron concentration observed in the isogenic deficiency mutants were significantly lower when compared to the WT strain containing the functional endogenous iron transport systems. These results showed c2515 and c2516 conferred a significant increase in uptake of ferric under iron-limited conditions, suggesting they may function as a ferric iron transporter. Thus, c2515, in combination with c2516, is critical for growth under low-iron conditions and this effect may be mediated by favoring siderophore production in APEC.

As bacterial cells require iron for basic cellular processes such as respiration, defects in cellular iron uptake and utilization would decrease bacterial viability (25). In this study, the chicken-derived macrophages were infected with the WT and its derivative mutants strains. At the indicated times following infection, the macrophages were lysed, and the bacterial viability was determined. The results showed that the bacterial proliferation in macrophages were impaired by mutation of the c2515 and/or c2516 genes, indicating that iron uptake is involved in stimulation of bacterial viability.

Although the pathogenic mechanism of APEC is complex, increasing evidence has demonstrated that iron acquisition systems are important for APEC infectivity (15, 16). The well-established chicken infection model was used to assess the relative and combined contributions of these genes to APEC pathogenesis. In the 1-day-old chicks lethality model, the double mutants exhibited lowered virulence than any single mutant, which are all less virulent than the WT strain. We next examined the ability of the mutants to colonize 3-week-old chickens. The c2515 and/or c2516 mutants showed significantly decreased colonization compared with the wild-type strain in various organs tested in a single-strain challenge model. The complementation strain restored the virulence, but it did not fully reach the levels of WT strain. It is possible that the reduced level of regain of the phenotypes may be due to poor or different expression of these genes in the complemented strains. As well, the complementation plasmid may be instability in the absence of selective antibiotic pressure. Without antibiotic pressure, the plasmid could be lost, resulting in a reduced level of complementation due to loss of the genes from a portion of the cell population. These results show that disruption of c2515 and c2516 may attenuate APEC virulence in chickens, indicating that they are required for systemic infection in the chicken infection model.

As the product of c2515 and c2516 are putative iron transporters, which are known to mainly partake in uptake ferric-siderophores from the periplasm into the cytoplasm. It is unclear how such genes could affect production of siderophores. The E516 strain produce four types of siderophores, including enterobactin, yersiniabactin, salmochelin and aerobactin, and use exogenous siderophores as well as citrate. It is important to know how or why loss of the c2515 and c2516 genes can contribute to decreased growth in iron-depleted medium and reduced virulence considering numerous iron systems are present in APEC E516. Through qRT-PCR analysis, we found that knockout of these genes somehow significantly reduced the expression of entA, fepC, and iutA, which implied that the enterobactin and aerobactin may be compromised or reduced in the double mutant. However, the concrete mechanism for these affections is unclear and need further study.

Taken together, our results indicate that c2515 and c2516 may involve in iron uptake and contributes to APEC virulence, suggesting the potential of these systems to act as targets for novel antimicrobial agents.

DATA AVAILABILITY STATEMENT

The original contributions generated in the study are included in the article-supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee of Yangzhou University [Approval ID: SYXK (Su) 2007–0005, September 21, 2016].

AUTHOR CONTRIBUTIONS

QG designed the experiments and drafted the manuscript. XL carried out the main experiments. SS performed the statistical analysis. LY participated in the chicken infection assays. SG reviewed the manuscript and gave instructions in this study. All authors contributed to the article and approved the final manuscript.

FUNDING

This work was supported by the National Key R&D Program of China (2017YFD0500203, 2017YFD0500705), National Natural Science Foundation of China (31602059, 31672553). This work was also supported by the General Financial Grant from the China Postdoctoral Science Foundation (2015MS80477), Jiangsu Postdoctoral Science Foundation (1501076C), and the project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

ACKNOWLEDGMENTS

We are grateful to Prof. Xin’an Jiao of the Jiangsu Key Laboratory of Zoonosis at Yangzhou University for kindly providing the HD11 cell line, and Prof. Guoqiang Zhu at Yangzhou university for kindly providing the plasmid pACYC184.
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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