Aerobactin Synthesis Genes \textit{iucA} and \textit{iucC} Contribute to the Pathogenicity of Avian Pathogenic \textit{Escherichia coli} O2 Strain E058

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Abstract

Aerobactin genes are known to be present in virulent strains and absent from avirulent strains, but contributions of \textit{iucC} and \textit{iucA}, which are involved in aerobactin synthesis, to the pathogenicity of avian pathogenic \textit{Escherichia coli} (APEC) have not been clarified. In this study, effects of double mutants (\textit{iucA/iutA} or \textit{iucC/iutA}) compared to those of single mutants (\textit{iucA}, \textit{iucC} or \textit{iutA}) of aerobactin genes on the virulence of APEC strain E058 were examined both \textit{in vitro} (aerobactin production, ingestion into HD-11 cells, survival in chicken serum) and \textit{in vivo} (competitive growth against parental strain, colonization and persistence). In competitive co-infection assays, compared to the E058 parental strain, the E058\textit{iucA} mutant was significantly reduced in the liver, kidney, spleen (all \(P<0.01\)), heart and lung (both \(P<0.001\)). The E058\textit{iutA} mutant was also significantly reduced in the liver, lung, kidney, all (\(P<0.01\)), heart and spleen (both \(P<0.001\)). The E058\textit{iucC} mutant was significantly attenuated in the heart and kidney (both \(P<0.05\)) and showed a remarkable reduction in the liver, spleen and lung (\(P<0.01\)); meanwhile, both E058\textit{iucA/iutA} and E058\textit{iucC/iutA} double mutants were sharply reduced as well (\(<0.001\)). In colonization and persistence assays, compared with E058, recovered colonies of E058\textit{iucA} were significantly reduced from the liver, lung, spleen and kidney (\(<0.01\)) and significantly reduced in the heart (\(<0.001\)). E058\textit{iutA} was significantly reduced from the heart, lung, liver, spleen, kidney and \((P<0.01)\). E058\textit{iucC}, E058\textit{iucA/iutA} and E058\textit{iucC/iutA} were significantly decreased in all organs tested (\(<0.01\)). These results suggest that \textit{iutA}, \textit{iucA} and \textit{iucC} play important roles in the pathogenicity of APEC E058.

Introduction

Although iron is an essential element for all living cells, ferric iron is barely soluble at biological pH. Therefore, iron can be taken up in a complexed form via siderophores, a process that has been shown to contribute to the pathogenicity of avian pathogenic \textit{Escherichia coli} (APEC) strains. The hydroxamate siderophore aerobactin has previously been associated with the pathogenicity of APEC strains [1,2,3,4,5,6]. The ability of virulent microorganisms to grow under iron-limiting conditions may promote systemic infections [7,8,9]. As determined by the lethality for 1-day-old chicks in avian strains of \textit{E. coli} [1], this physiological trait is highly correlated with APEC virulence. Lafont et al. stated that aerobactin genes are present in virulent strains but absent from avirulent strains, which has been confirmed by epidemiological studies [3]. Work undertaken by Tivendale et al. found that the \textit{iucA} gene located on the pVM01 plasmid of APEC is highly correlated with virulence, adding support to the hypothesis that aerobactin is a virulence factor in APEC strains [10]. Dozois et al. revealed that \textit{iutA} is dedicated to the virulence of APEC strain \(\chi7122\) [11]. In the APEC O2 strain, the aerobactin operon is situated on the pAPEC-O2-ColV plasmid [12], which contains genes responsible for the synthesis of the hydroxyl siderophore aerobactin (\textit{iucABCD}) and for ferric aerobactin uptake (\textit{iutA}) [13,14,15]. Among APEC strains, iron acquisition systems can be encoded by plasmid genes [16,17] or by chromosomal pathogenicity islands [18]. Aerobactin is a hydroxamate siderophore that is encoded by a plasmid operon [19,20]. Hence, the expression of aerobactin should be associated with virulence of APEC. The aerobactin operon, which includes \textit{iucABCD} and \textit{iutA}, is localized to the APEC ColV plasmid [21].

Based on our previous observation that the \textit{iucCD} gene of APEC E058 is up-regulated \textit{in vivo} and the \textit{iutA} transcription was also observed up-regulated in a microarray to detect the transcriptoms of avian pathogenic \textit{Escherichia coli} (APEC) grown \textit{in vivo} compared with those \textit{in vitro} [22], we hypothesized that \textit{iutA} or \textit{iucC} and/or \textit{iucD} is involved in the virulence of APEC E058. To our knowledge, the contribution of \textit{iucC} to the pathogenesis of APEC and the effects of double mutations in \textit{iucA/iutA} or \textit{iucC/iutA} on APEC virulence compared to that of the each single mutation have not been reported. In this study, we chose to delete the single \textit{iucC}, \textit{iucA} or \textit{iutA} gene or the double \textit{iucC/iutA} or \textit{iucA/iutA} genes to investigate their pathogenic roles in APEC O2 strain E058. A series of pathogenicity tests, including bactericidal assay in chicken...
serum, HD-11 cells ingestion assay, colonization and persistence in chickens, and in vivo competition assay, were employed to evaluate the pathogenicity of the APEC E058 iucA, E058 iucC, E058 iutA, E058 iucA iutA and E058 iucC iutA mutants compared with the E058 parent strain.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Research Council. All experiments and procedures performed on the animals were approved by the Animal Care and Use Committee of Yangzhou University (approval ID: SYXK (Su) 2007-0005). Chickens were provided with food and water ad libitum. After 24 h of infection, the birds were euthanized by carbon dioxide asphyxiation and then dissected with aseptic surgical techniques.

Bacterial strains, plasmids, primers, media and growth conditions

The strains, plasmids and cell line used in this study are listed in Table 1. The primers applied in this study are listed in Table 2. Cells were routinely grown at 37°C in Luria-Bertani (LB) broth or on LB agar with/without appropriate antibiotics: kanamycin (Km) (50 µg/ml), zeocin (25 µg/ml) or ampicillin (Amp) (60 µg/ml) unless otherwise specified. The chicken macrophage cell line HD-11 was maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS, PAA, Pasching, Australia) at 37°C, 5% CO2.

DNA and genetic manipulations

All restriction and DNA-modifying enzymes and a 200 bp DNA marker purchased from Takara (Dalian, Liaoning, China) were used according to the supplier’s recommendations. The Lambda DNA/EcoR I-plus-Hind III molecular size standard was purchased from Fermentas (Shenzhen, Guangdong, China). Purification of PCR products and DNA fragments was performed using kits manufactured by Promega (Madison, WI, USA). The DIG High Prime DNA Labeling and Detection kit was purchased from Roche (Indianapolis, IN, USA). Transformation of E. coli strains was routinely carried out using electroporation. DNA and deduced amino acid sequence analyses were performed using DNAStar Lasergene software to predict conserved domains and using the search engine at http://blast.ncbi.nlm.nih.gov/Blast.cgi. DNA nucleotide sequences were determined by Sangon Co. LTD. (Shanghai, China). Reactions were carried out using Taq DNA polymerase (Fermentas) under the following conditions: 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min and finally an extension at 72°C for 10 min.

Construction of iutA, iucA, iucC, iucA/iutA and iucC/iutA mutant strains

The iucA, iucC and iutA genes were amplified from template which was a boiled preparation of the overnight culture of E058 using primer pairs iucA-F/iucA-R, iucC-F/iucC-R and iutA-F/iutA-R respectively (Table 2), and the PCR products were cloned into pBluescript SK (+) to form pS-iucA, pS-iucC and pS-iutA respectively. To form pS-iucA-Zeo, the iucA PCR product, which was amplified from pS-iucA using primers pS-iucA-F/R (Table 2) and digested with EcoRI/EcoRI V, was ligated with the Zeo’ gene cartridge originating from pEM7/Zeo digested with the same enzymes. To form pS-iucC-Zeo, the pS-iucC fragment digested with BamH I was ligated with the Zeo’ gene cassette amplified from pEM7/Zeo using primers Zeo-BamHI F/R and digested with BamH I. To form pS-iutA-Kan, the pS-iutA plasmid and the pUC4K plasmid (containing the Kan’ gene) were both digested with Pst I and ligated together to form pS-iutA-Kan. Deletions of target genes iucA, iucC, iutA, iucA/iutA and iucC/iutA in the APEC O2 E058 strain essentially were performed as described by Xiong et al. [23].

Complementation of mutants with the native iucA or iucC gene

For the complementation study, the native genes iucA (1725 bp) and iucC (1743 bp) were handled similarly by amplification using primers Re-iucA-F/R and Re-iucC-F/R (Table 2), with the introduced restriction enzyme recognition sites BamHI EcoRI and EcoRI I-SalI, respectively. To determine that the sequences were in frame, the iucA and iucC fragments were inserted into the pMD818-T simple vector and sequenced by Sangon Co. LTD. (Shanghai, China). The iucA and iucC PCR products and expression vector pGEX-6P-1 were digested with BamHI EcoRI and EcoRI I-SalI, respectively, and ligated using T4 DNA ligase for 4 h at 22°C. The ligation mix was then transformed into DH5α and plated on LB agar plates containing ampicillin. Colonies were tested for the presence of iucA and iucC using standard primers iucA-F/iucA-R and iucC-F/iucC-R (Table 2), respectively. The modified plasmid pGEX-6P-1 with the iucA or iucC insert was isolated from DH5α and electroporated into E058 iucA or E058 iucC to complement the gene deleted.

RT-PCR analysis

To extract total RNA from each strain, 1 ml of bacteria culture was collected by centrifugation. RNA was extracted by using the RNAeasy Mini kit purchased from QIAGEN (Qiagen, Düsseldorf, Germany) 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). Primer sets for PCR amplification of target genes shiG, iucA, iucB, iucC, iucD, iucK and O2-ColV16 in cDNA samples are listed in Table 2. In parallel, PCRs were performed with pAPEC-O2-ColV-like plasmid DNA as a positive control and cDNA samples without activation of the reverse-transcription (RT) as a negative control. The PCR products were resolved on 0.8% agarose gels and visualized by ethidium bromide staining.

Growth in LB and under iron limitation

Each strain was cultured in LB medium with the appropriate antibiotic at 37°C overnight. The next day the cell density was estimated by spectrophotometry, and cultures were diluted in PBS prior to inoculation in LB medium with or without 200 µM 2,2'-dipyridyl (DIP) to achieve an approximate starting concentration of 103 to 104 colony-forming units (CFUs) per ml, which was confirmed by viable counts. Mixtures were incubated at 37°C with shaking, and aliquots of these cultures were removed at set time intervals for use in determining viable counts. The data represent averages of three independent assays.

Bactericidal activity of specific-pathogen-free (SPF) chicken serum

The serum bactericidal assay was performed in a 96-well plate essentially as described previously [24] but with the following modifications. Briefly, complement-sufficient serum was prepared and pooled from SPF chickens (White Leghorn, Jinan SPAFAS
The birds were treated in the experiments in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (Approved by the State Council on October 31, 1988).

Animal infection models

A competitive co-infection model and a comparative single-strain infection model were used to investigate the contribution of the iucA, iucC, iutA/iucA and iucC/iutA genes to the virulence of APEC E058. Animals used in these studies were White Leghorn SPF chickens obtained from Jinan SPAFAS Poultry Co. LTD., Shandong, China). The chicken serum was diluted to 0.5, 2.5, 5, 12.5 and 25% 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 (25%) heat-inactivated SPF chicken serum or PBS alone and 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.

Animal experiments were also carried out to determine the colonization ability of the E058 iucA, E058 iucC, E058 iutA, E058 iucA iutA and E058 iucC iutA mutant strains in comparison with the E058 parent strain. For this single-strain infection model, 3-week-old White Leghorn chickens (15 chickens per group) were inoculated in the left thoracic air sac with 0.1 ml (10^6 CFU) of a suspension containing the wild-type E058 strain or mutant derivatives. After 24 h, all chickens were euthanized and serial dilutions were plated on LB medium with or without kanamycin or zeocin for selection of the mutant or total bacteria [25].

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**Table 1. Strains, plasmids and cell line used in this study.**

| Strains                     | Description                                      | Source               |
|-----------------------------|--------------------------------------------------|----------------------|
| E058                        | Wild-type avian E. coli serotype O2               | [44]                 |
| DH5α                        | endA1 hsdR17 (r1, mcrA) supE44 thi-1 recA1 gyrA (Nal^R) RelA1 Δ (lacI2ΔZamF-U169deoR) U169deoR (pBR322 lacZΔ (lacI2) M15) | Invitrogen           |
| LG1522                      | Aerobactin indicator strain                      | [5]                  |
| E058ΔiucA                   | iucA mutant of E058, Kan^R                       | This study           |
| E058ΔiucC                   | iucC mutant of E058, Zeo^R                       | This study           |
| E058ΔiucAΔiucA              | iucA and iucA double mutant of E058, Zeo^R, Kan^R| This study           |
| E058ΔiucC                   | iucC mutant of E058, Zeo^R                       | This study           |
| E058ΔiucCΔiucA              | iucA and iucC double mutant of E058, Zeo^R, Kan^R| This study           |
| Re-E058ΔiucA                | Complementation of E058ΔiucA                     | This study           |
| Re-E058ΔiucC                | Complementation of E058ΔiucC                     | This study           |

| Plasmids                    | Description                                      | Source               |
|-----------------------------|--------------------------------------------------|----------------------|
| pMD^®18-T simple vector     | TA cloning vector, Ap^R                          | Promega              |
| pT-iucA                     | iucA cloned into pMD^®18-T simple vector          | This study           |
| pT-iucC                     | iucC cloned into pMD^®18-T simple vector          | This study           |
| pBluescript II SK(-)         | Cloning vector                                   | Fermentas            |
| pS-iucA                     | Hind III-BamHI iucA fragment cloned into SK(-)   | This study           |
| pS-iucC                     | EcoRI Xba I iucC fragment cloned into SK(-)       | This study           |
| pEM7/Zeo                    | Zeocin-resistant cassette                         | Invitrogen           |
| pUC4K                       | Kanamycin-resistant cassette                      | Invitrogen           |
| pS-iucA-Zeo                 | Zeocin-resistant gene inserted into pS-iucA      | This study           |
| pS-iucC-Zeo                 | Zeocin-resistant gene inserted into pS-iucC      | This study           |
| pGEX-6P-1                   | Bacterial expression vector                      | Amersham             |
| pGEX-6P-1-iucA              | iucA cloned into pGEX-6P-1 vector                | This study           |
| pGEX-6P-1-iucC              | iucC cloned into pGEX-6P-1 vector                | This study           |

| Cell line                   | Description                                      | Source               |
|-----------------------------|--------------------------------------------------|----------------------|
| HD-11                       | Chicken macrophage line, chicken myelomonocytic transformed by the myc-encoding MC29 virus | [45]                 |

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poultry Co. LTD., Jinan, Shandong, China). The chicken serum was diluted to 0.5, 2.5, 5, 12.5 and 25% 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 (25%) heat-inactivated SPF chicken serum or PBS alone and 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.

Animal infection models

A competitive co-infection model and a comparative single-strain infection model were used to investigate the contribution of the iucA, iucC, iutA/iucA and iucC/iutA genes to the virulence of APEC E058. Animals used in these studies were White Leghorn SPF chickens obtained from Jinan SPAFAS Poultry Co. LTD. The birds were treated in the experiments in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (Approved by the State Council on October 31, 1988).

Briefly, for competitive co-infection assays, fifteen 3-week-old chickens were infected intratracheally with equal doses (1×10^7 CFU each) of the wild-type strain E058 or the mutant strains E058ΔiucA, E058ΔiucC, E058ΔiutA, E058ΔiucAΔiutA or E058ΔiucCΔiutA. At 24 h post-infection, the spleen, heart, liver, lung and kidney of inoculated chickens were collected, weighed and homogenized, and serial dilutions were plated on LB medium with or without kanamycin or zeocin for selection of the mutant or total bacteria [25].

Animal experiments were also carried out to determine the colonization ability of the E058ΔiucA, E058ΔiucC, E058ΔiutA, E058ΔiucAΔiutA and E058ΔiucCΔiutA mutant strains in comparison with the E058 parent strain. For this single-strain infection model, 3-week-old White Leghorn chickens (15 chickens per group) were inoculated in the left thoracic air sac with 0.1 ml (10^6 CFU) of a suspension containing the wild-type E058 strain or mutant derivatives. After 24 h, all chickens were euthanized and examined for macroscopic lesions. The heart, liver, spleen, lung and kidney of the chickens were collected, weighed, suspended in PBS and homogenized. Bacterial loads were determined by plating serial dilutions of the homogenates on selective LB agar medium [26].

**HD-11 ingestion assay**

For ingestion assays, avian macrophage HD-11 cells were grown in DMEM with 10% FBS at 37°C with 5% CO_2 at 2×10^5 cells per well in 24-well cell culture plates and incubated for 24 h
Table 2. Primers designed and used in this study.

| Primer name | Sequence 5′-3′ | Position (bp) | Source |
|-------------|----------------|---------------|--------|
| iutA-F      | CGAAGCTTTCTCAACCCACTGCTTCTT (iutA sense; Hind III site underlined) | 33–51 | This study |
| iutA-R      | TTAGGATCCGTGATGTTGACATGACGACCTT (iutA antisense; BamHI site underlined) | 1981–1999 | This study |
| iucA-F      | CTCGAAGCTTCTAGGCTCTCCATGATGATTGCT (iucA sense; Hind III site underlined) | 44–61 | This study |
| iucA-R      | CTGCCGAATCTAGGCTCTCCATGATGATTGCT (iucA antisense; BamHI site underlined) | 1538–1573 | This study |
| pS-iucA-F   | CTGGATATCTCGGAGAAGATCTGCATAC (pS-iucA sense; EcoR V site underlined) | 492–509 | This study |
| pS-iucA-R   | CTGGATATCTGAGCAGAAGATCTGCATAC (pS-iucA antisense; EcoR I site underlined) | 1200–1217 | This study |
| iucC-F      | CTGGATATCTGAGAAGATCTGCATAC (iucC sense; EcoR I site underlined) | 14–31 | This study |
| iucC-R      | CTCTCGAATCTGAGAAGATCTGCATAC (iucC antisense; Xba I site underlined) | 1715–1732 | This study |
| Zeo-BamHI-F | CTCGGATCCACAGGTGATGTTGACATAAT (Inserting zeocin gene; BamHI I site underlined) | 1938–1955 | This study |
| Zeo-BamHI-R | CTCGGATCCACAGGTGATGTTGACATAAT (Inserting zeocin gene; BamHI I site underlined) | 2366–2383 | This study |
| GF          | CACAGTCTTACTGGCAGT (shig gene) | 114–131 | This study |
| GR          | TTCTCGGATATGGACAG (shig antisense) | 266–283 | This study |
| BF          | TTGCTGACGAGCAATGGGC (iucB sense) | 701–718 | This study |
| BR          | ACCTCGAGAAGATCTGGTA (iucB antisense) | 921–938 | This study |
| DF          | ATGGCATCAGTGGCCATT (iucD sense) | 782–799 | This study |
| DR          | TACGGTACGACTTCCTGAT (iucD antisense) | 1181–1198 | This study |
| O2F         | ATGGCTGTCTTGGTTGCTCA (O2ColV sense) | 1–19 | This study |
| O2R         | TCATGAAAGTTGGCAAGCATC (O2ColV antisense) | 204–222 | This study |
| Re-iucA-F   | TCAGATGATCCATGATTGCCCTGGA (iucA sense; BamHI site underlined) | 1–18 | This study |
| Re-iucA-R   | TCAGATGATCCATGATTGCCCTGGA (iucA antisense; EcoR I site underlined) | 1708–1725 | This study |
| Re-iucC-F   | CGCGAATCTGAGCAGAAGATCTGG (iucC sense; EcoR I site underlined) | 1–18 | This study |
| Re-iucC-R   | CGCGAATCTGAGCAGAAGATCTGG (iucC antisense; Sal I site underlined) | 1726–1743 | This study |

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Statistical analyses
Statistical analyses for in vivo tests were performed using GraphPad Prism v5.0 software package (GraphPad Software). The Wilcoxon matched-pair test was used to analyze data from the competition assay. The Mann Whitney U test was performed for analysis of results from the colonization and persistence assay.

Results
Construction of the iutA, iucA and iucC knockout mutants
The iucA and iucC genes situated on the pAPEC-O2-ColV-like plasmid from APEC E058 were sequenced and confirmed to be 1725 bp and 1743 bp in length, respectively. The putative iutA gene in the wild-type strain E058 was identified by BLAST searches of the E. coli plasmid pAPEC-O2-ColV complete sequence. Nucleotide sequence analysis showed that the iutA DNA fragment was 1966 bp in length. The APEC E058 mutants were created by the method described by Xiong et al. [25]. Sequence analysis revealed that the shig gene encoding a conserved hypothetical protein is upstream of iucA. The iucB gene encoding the aerobactin biosynthesis protein IucB is downstream of iucA and upstream gene of iucC. The iucD gene, encoding the aerobactin IucD protein, is downstream of iucC. The iucD gene is downstream of iucD and upstream of the O2-ColV16 gene.

The iucA and iucC knockout mutants were constructed by allelic exchange with an insertion of a Zeo′ cassette, while the iutA knockout mutant was made with an insertion of a Kan′ cassette. By sequence analysis of the PCR products, the iucA and iucC genes were confirmed to have been replaced by the Zeo′ gene in the pAPEC-O2-ColV-like plasmid of APEC E058 at the predicted position.

Pathogenicity of Aerobactin Synthesis Genes

Aerobactin production
Mutants were also assessed for aerobactin production as described by Vidotto et al. [5]. Low-iron agar assay plates, composed of M-9 minimal salts, containing 200 μM 2,2′-dipyridyl and 0.2% glucose, were seeded with 1 ml/liter of an overnight culture of the indicator organism, E. coli LG1522, which is incapable of producing aerobactin, but can use exogenously produced aerobactin. The E058 strain and its mutants were stab inoculated into the agar, and the plates were incubated at 37°C for 24 h. Following incubation, plates were observed for growth of the indicator organism around the stabs in a halo as evidence of aerobactin elaboration by the test mutants.

prior to ingestion assays. Bacteria were inoculated into cells at a multiplicity of infection (MOI) of 100. Inoculated cells were incubated at 37°C with 5% CO2 for 1 h under to allow the bacteria to be ingested by the cells. Thereafter, the cells were washed with PBS, and the extracellular bacteria were eliminated by culturing in DMEM medium containing gentamicin (100 μg/ml) at 37°C for 1.5 h prior to washing the cells again using PBS. The intracellular bacteria were treated with 1 ml 0.1% Triton X-100, and a 100 μl aliquot of this suspension was inoculated into 900 μl PBS. Serial dilutions (1:10) of each sample were plated onto LB agar plates. The resulting colonies were counted after 24 h of incubation. Wells containing only HD-11 cells were used as negative controls. The assay was performed in triplicate. The ingestion ratio was determined by dividing the number of ingested bacteria by the number of bacteria in the initial inoculation.
position, and the generated mutants were named E058\(\Delta\)iucA and E058\(\Delta\)iucC, respectively. The \(iuc\) gene was also confirmed to have been replaced by the \(\mathrm{Kan}^+\) gene in the pAPEC-O2-ColV-like plasmid of APEC E058 at the predicted position, and the generated mutant was named E058\(\Delta\)iuc.

The \(iuc\) gene mutant plasmid containing the replacement Zeo\(^R\) gene was PCR amplified with primers iuc-A-F and iuc-A-R (Table 2) and purified for electroporation to E058\(\Delta\)iucA competent cells as described previously [23]. After 18 h of incubation, the resulting Zeo\(^R\) \(\mathrm{Kan}^+\) colonies were selected for PCR identification using primers iuc-A-F and iuc-A-R or iutA-F and iutA-R (Table 2) and analyzed by sequencing. The \(iuc\) and \(iutA\) double mutant was named E058\(\Delta\)iucA\(\Delta\)iutA. The \(iuc\) and \(iutA\) double mutant E058\(\Delta\)iucA\(\Delta\)iutA was generated using the same method.

RT-PCR analysis

To determine whether the insertion had a polar effect on the upstream or downstream genes, total RNA samples extracted from the parental E058 and mutants E058\(\Delta\)iucA, E058\(\Delta\)iucC, E058\(\Delta\)iucA\(\Delta\)iutA and E058\(\Delta\)iucA\(\Delta\)iutA were analyzed by RT-PCR using primer sets designed for \(\mathrm{shift}\) [GF/GR], \(iuc\) [iuc-A-F/iuc-A-R], \(iucB\) [BF/BR], \(iuc\) [iuc-C-F/iuc-C-R], \(iucD\) [DF/DR], \(iuc\) [iuc-A-F/iutA-R] and O2-ColV16 [O2F/O2R] (Table 2). When compared to the parental E058, the insertion of the \(\mathrm{Zeo}^R\) gene in the E058\(\Delta\)iucA, E058\(\Delta\)iucA\(\Delta\)iutA, E058\(\Delta\)iucC or E058\(\Delta\)iucA\(\Delta\)iutA mutant only transcribed the transcription of the \(iuc\) or \(iuc\) gene (Figure 1A, 1B, lane 10; 1D, 1E, lane 10). Meanwhile, insertion of the \(\mathrm{Kan}^+\) gene in the E058\(\Delta\)iucA\(\Delta\)iutA or E058\(\Delta\)iucA\(\Delta\)iutA mutant only transcribed the transcription of the \(iuc\) gene (Figure 1C, 1F, lane 10), and the upstream and downstream genes of all three target genes [\(iucA\), \(iucC\), \(iutA\)] were not influenced.

Complementation studies and the result of aerobactin production

For complementation studies, the native \(iuc\) gene was amplified using primers Re-iuc-A-F/Re-iuc-A-R (Table 2) and subcloned into the pGEX-6P-1 vector by digestion with restriction enzymes BamHI and EcoR I and ligation. The recombinant plasmid pGEX-6P-1-iuc was electroporated into E058\(\Delta\)iuc to complement the deleted \(iuc\) gene, and colonies on LB agar containing ampicillin were tested for the presence of \(iuc\) using primers Re-iuc-A-F/Re-iuc-A-R (Table 2). A positive clone was named Re-E058\(\Delta\)iucA. Using the same method, Re-E058\(\Delta\)iucC was generated except that the restriction enzymes were EcoR I and Sal I. In testing for aerobactin production, the wild-type strain E058, the mutant E058\(\Delta\)iucA and complementation strains Re-E058\(\Delta\)iucA and Re-E058\(\Delta\)iucC showed the ability to produce aerobactin, while the E058\(\Delta\)iucA, E058\(\Delta\)iucA\(\Delta\)iutA, E058\(\Delta\)iucC and E058\(\Delta\)iucA\(\Delta\)iutA mutants lost the ability to produce aerobactin (Data not shown).

Characterization of the mutants

Growth curves, colonies, bactericidal assay and ingestion capability of the E058\(\Delta\)iucA, E058\(\Delta\)iucC, E058\(\Delta\)iucA\(\Delta\)iutA and E058\(\Delta\)iucA\(\Delta\)iutA mutants were similar to those of the parental strain E058. Compared to the parent strain, no significant differences in the growth during the logarithmic phase were observed for all five mutants grown in LB rich medium and under iron limitation (Figure 2). Results of the bactericidal assay showed that the abilities of the E058\(\Delta\)iucA, E058\(\Delta\)iucC, E058\(\Delta\)iucA\(\Delta\)iutA, E058\(\Delta\)iucC and E058\(\Delta\)iucA\(\Delta\)iutA mutants to survive in SPF chicken serum were not affected (Figure 3). The APEC E058 mutants (E058\(\Delta\)iucA, E058\(\Delta\)iucC, E058\(\Delta\)iucA\(\Delta\)iutA, E058\(\Delta\)iucA and E058\(\Delta\)iucC) all exhibited a similar phenotype to the parental strain E058. Compared to the parent strain, no significant differences in the growth during the logarithmic phase were observed for all five mutants grown in LB rich medium and under iron limitation (Figure 2). Results of the bactericidal assay showed that the abilities of the E058\(\Delta\)iucA, E058\(\Delta\)iucC, E058\(\Delta\)iucA\(\Delta\)iutA, E058\(\Delta\)iucC and E058\(\Delta\)iucA\(\Delta\)iutA mutants to survive in SPF chicken serum were not affected (Figure 3). The APEC E058 mutants (E058\(\Delta\)iucA, E058\(\Delta\)iucC, E058\(\Delta\)iucA\(\Delta\)iutA, E058\(\Delta\)iucA and E058\(\Delta\)iucC) all exhibited a similar phenotype to the parental strain E058.
from E058ΔiucCΔiutA without activation of RT. Lanes 2, 4, 6: pAPEC-O2-ColV-like DNA from E058. Lanes 9, 11, 13: pAPEC-O2-ColV-like DNA from E058ΔiucAΔiutA. Primers: Lanes 1, 2, 8, 9: DF/DR, Lanes 3, 4, 10, 11: iutA-F/iutA-R. Lanes 5, 6, 7, 12, 14: O2F/O2R (Table 2). A 200 bp marker (Takara) was used as the molecular size standard (M).

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E058ΔiucCΔiutA had ingestion ratios (0.23%, 0.24%, 0.19%, 0.23% and 0.20%, respectively) which were not apparently different from that of the parental E058 (0.23%). Thus, the deletion of either the single gene of iutA, iucA or iucC, the double genes of iucA/iutA or iucC/iutA had no effect on the ingestion of APEC E058 into HD-11 cells.

Deletions of iutA, iucA, iucC, iucA/iutA and iucC/iutA attenuate APEC E058 virulence in vivo

To determine the effects of iutA, iucA, iucC, iucA/iutA and iucC/iutA on the virulence of APEC E058 in vivo, competitive co-infections were carried out. As shown in Figure 4, at 24 h post-challenge, the E058ΔiucC mutant was significantly attenuated in the liver, lung and kidney (P<0.001) and was attenuated to an even greater degree in the heart and spleen (P<0.001) (Figure 4). The E058ΔiucA mutant was significantly attenuated in the liver, spleen and kidney (P<0.01), and especially in the heart and lung (P<0.001) (Figure 4). The E058ΔiucC mutant was also significantly attenuated in the heart and kidney (P<0.05) and exhibited more significant levels of attenuation in the liver, spleen and lung (P<0.01). Both double mutants (E058ΔiucAΔiucC and E058ΔiucC-

\[ \text{iutA} \]) were significantly attenuated in all five tested tissues (P<0.001) (Figure 4).

When birds were infected with the E058ΔiutA, E058ΔiucA, E058ΔiucCΔiutA, E058ΔiucC and E058ΔiucAΔiutA mutants, distinct reductions in the number of bacteria recovered from all tested tissues were observed compared to those of birds infected with E058. At 24 h after infection with the E058 parent strain, maximum colonization was observed in the lung (1.1×10^5 CFU.g^-1), and minimal colonies were obtained in the heart (8.7×10^3 CFU.g^-1), while colony numbers in the spleen (6.3×10^4 CFU.g^-1), liver (2.1×10^4 CFU.g^-1) and kidney (3.2×10^4 CFU.g^-1) were intermediate (Figure 5). Compared to the E058 wild-type strain, the mutant E058ΔiucC colony numbers were significantly decreased in the lung (6.5×10^4 CFU.g^-1) (P<0.01), heart (70 CFU.g^-1) (P<0.01), kidney (2.6×10^4 CFU.g^-1) (P<0.01), liver (5.3×10^4 CFU.g^-1) (P<0.01) and spleen (5.9×10^4 CFU.g^-1) (P<0.01) and colonization with the E058ΔiucA mutant was significantly lower in the lung (7.8×10^3 CFU.g^-1) (P<0.01), spleen (6.8×10^3 CFU.g^-1) (P<0.01), heart (12 CFU.g^-1) (P<0.001), liver (8.1×10^3 CFU.g^-1) (P<0.01) and kidney (9.0×10^3 CFU.g^-1) (P<0.01) (Figure 5). Levels of the E058ΔiucAΔiucC mutant colonized in the lung (3.6×10^4 CFU.g^-1), spleen (1.02×10^3 CFU.g^-1), heart (14 CFU.g^-1), liver (82 CFU.g^-1) and kidney (96 CFU.g^-1) were sharply decreased compared to those of the E058 parent strain (P<0.001) (Figure 5). Interestingly, numbers of the E058ΔiucC mutant in the lung (4.3×10^4 CFU.g^-1), spleen (6.5×10^4 CFU.g^-1), heart

\[ \text{iucC} \]...
(9 CFU.g⁻¹), liver (67 CFU.g⁻¹) and kidney (64 CFU.g⁻¹) also dropped dramatically compared to those of E058 (P < 0.001) (Figure 5). Not surprisingly, the E058ΔiucAΔiutA mutant colonized in the lung (4.2 × 10² CFU.g⁻¹), spleen (1.3 × 10³ CFU.g⁻¹), heart (17 CFU.g⁻¹), liver (90 CFU.g⁻¹) and kidney (93 CFU.g⁻¹) also decreased remarkably compared to E058 (P < 0.001) (Figure 5). On average the mutant strains were reisolated at levels of 10–1000 times lower than the wild-type strain from the internal organs tested at 24 h post-challenge. These results indicated that iutA and both iucA and iucC genes are involved in the process of systemic infection of APEC E058.

Figure 3. Bactericidal activity of SPF chicken serum against the wild type strain and mutants. Strains E058 (black bar), E058ΔiutA (white bar), E058ΔiucA (gray bar), E058ΔiucAΔiutA (gray dots), E058ΔiucC (gray oblique lines), E058ΔiucCΔiutA (gray grids). HI represents the group of heat-inactivated 25% SPF chicken serum used as controls for each strain tested. Data represent averages of three independent assays. doi:10.1371/journal.pone.0057794.g003

Figure 4. Competition assays between wild-type E058 and mutants E058ΔiutA, E058ΔiucA, E058ΔiucC, E058ΔiucAΔiutA or E058ΔiucCΔiutA inoculated simultaneously. Negative competitive index (CI) values indicate a decreased capacity of the mutants to compete for growth with the wild-type strain. Horizontal bars indicate the mean log₁₀CI values. Each data point represents a sample from an individual chicken. Statistically significant differences in CI values between E058 and its mutants are indicated with asterisks (* P < 0.05, ** P < 0.01, *** P < 0.001). doi:10.1371/journal.pone.0057794.g004
Discussion

Many types of bacteria have several iron uptake systems to survive and grow in different host environments [26]. In the virulence assay, the mutants were significantly attenuated in the observed tissues. Sequestration of iron results in an extremely low free iron concentration that would limit bacterial growth in this complicated environment in vivo [25]. The ability to obtain iron from the host determines the pathogenicity of APEC strains and may be increased by the aerobactin and iron system [27].

Iron has many vital functions in bacteria and is a cofactor for a large number of enzymes [28]. The enterochelin system is one of the iron uptake systems that is chromosomally encoded and appears less able to compete with transferrin in vivo, although it is very efficient in vitro [29]. By contrast, the aerobactin system is considered to be efficient in vivo and is involved in the invasive properties of human enteroinvasive E. coli and Shigella species [30,31]. The aerobactin operon encoded by either plasmids or chromosomes in several species of enteric organisms [6,15,20,31,32,33,34,35] contains genes responsible for the synthesis of the hydroxamate siderophore aerobactin (iuc) and for ferric aerobactin uptake (iut) [13,14,35]. Both components are strongly induced under iron starvation. Because aerobactin genes are present in virulent strains and absent from avirulent strains [3], they are regarded to play independent roles in the pathogenicity of APEC, especially in deep tissue damage, and its persistent infection [11]. The five genes encoding aerobactin are localized to an operon, which is present in the ColV plasmid [17]. The iucC gene is related to the aerobactin synthetase reaction, whereas the deletion of iucA will result in a severe polar effect [36]. A previous study from our laboratory [23] and independent reports from others [21,37] have provided a general overview of the aerobactin operon and several iron uptake system [38,39,40,41]. To date, many studies have focused on the characterization of the iuc genes [29,35,36,42], but the relationship between the iuc gene and the pathogenicity of APEC has not been clarified.

In this study, the E058\textit{ΔiucA}, E058\textit{ΔiucC}, E058\textit{ΔiucAΔiutA} and E058\textit{ΔiucCΔiutA} mutants with deletions of the \textit{iucA}, \textit{iucC}, \textit{iucA/iutA} and \textit{iucC/iutA} genes, respectively, lost the capacity to produce aerobactin, while the complementation of E058\textit{ΔiucA} and E058\textit{ΔiucC} restored their ability to produce aerobactin similar to the E058 parent strain. Meanwhile, the mutant E058\textit{ΔiutA} also showed the capacity to produce aerobactin. In the acquisition of iron, APEC O2 is known to contain at least two other chromosomal operons (yersinabactin and enterobactin) [17]. Although we deleted the aerobactin receptor gene \textit{iutA}, the other two chromosomal operons (yersinabactin and enterobactin) may have helped to compensate for the loss of \textit{iutA}. In addition, \textit{iucA} or \textit{iucC} is responsible for the synthesis of the hydroxamate siderophore aerobactin. While the deletion of one or both of these two genes may have resulted in the failure to produce aerobactin (hydroxamate type siderophore aerobactin), it may not have influenced the other two chromosomal operons (yersinabactin and enterobactin) in the acquisition of iron.

In LB broth and under conditions with limited iron, there was no obvious difference in the growth of the mutants and wild-type parent. During the assays of bacterial ingestion into HD-11 cells, no obvious difference was observed between the wild-type E058 and its mutants. These findings correspond to results of a previous study on E058\textit{ΔiucB} and E058\textit{ΔiucBAΔiutA} [23].
In the single-strain infection model, compared to E058, colonization levels of the E058ΔiucA and E058ΔiutA mutants were reduced in all five tested tissues (P<0.01 or P<0.001), and that of the E058ΔiucC mutant was remarkably reduced in the above-mentioned tissues (P<0.001). The same results were observed for both E058ΔiucAΔiutA and E058ΔiucCiutA double mutants.

The capacity of the E058ΔiutA, E058Δiuc, E058ΔiucΔiutA, E058ΔiucC and E058ΔiucΔiutA mutants to compete for growth in 3-week-old SPF chicken tissues with the E058 wild-type strain was also evaluated in a co-infection model. Compared to its parental strain, the E058ΔiutA mutant showed a significant reduction of bacterial counts in the heart, liver, kidney, spleen and lung (P<0.05 or P<0.01). The E058Δiuc mutant showed a remarkable reduction in all the tissues mentioned above (P<0.01 or P<0.001). The E058ΔiucC mutant was also significantly attenuated in all the tissues mentioned above (P<0.05 or P<0.01). Furthermore, highly significant reductions of both the E058ΔiucΔiutA and E058ΔiucC-ΔiutA double mutants were observed in all five tested tissues (P<0.001). The co-infection assay demonstrated that the deletion of either the iuc, iucA or iucC gene reduced the virulence of the APEC strain E058. However, it seemed a greater degree of colonization defects in an independent challenge versus a co-challenge experiment. As the individual infection results showed a 2-log decrease in colonization for most of the mutants in most tissues (10–1000-fold). Whereas, the co-challenge experiment showed a 2-log decrease in colonization for most of the mutants in most tissues (10–1000-fold). Whereby, the co-challenge experiment results showed a 1-log fitness defect for most mutants in all tissues. These results implied that the exogenous siderophores synthesized by the wild-type strain may be to a certain extent complement the effect of the siderophore synthesis mutations in a co-infection model. Similarly, a AucBΔEntD double mutant, defective in synthesis of both siderophores, was rescued by co-infection with a wild-type strain in the mouse UTI model [43].

The pathogenicity of mutants with deletion of either the whole iucABCDiutAD operon or the single gene iutA in APEC χ7122 is attenuated [11]. Meanwhile, the effect of the single gene iucA or iucC, and the double genes iucA/iutA or iucC/iutA on the virulence of APEC remains to be determined. Tivendale et al. demonstrated that the iucA gene could be amplified only from the three most virulent strains, E3, E30 and E956, but not from the less virulent strains, E135, E1043 and E1292. As discussed by these authors, the identification of potential virulence genes in avian colibacillosis can be attempted by using different approaches, such as to generate isogenic mutants of these genes and determine their virulence in birds [16]. In this study, we used this approach to explore the effects of iucA and iucC genes on the pathogenicity of the APEC strain E058. In a previous study, we demonstrated that either the iucB gene or iucA gene is likely involved directly or indirectly in iron uptake, with no obvious synergistic effect between these two genes, as it relates to the pathogenicity of APEC E058 [23]. To our knowledge, the present study is the first to report the contribution of iucC to the pathogenesis of APEC and the effects of double mutations in iucA/iucD or iucC/iucD on APEC virulence compared to that of each single mutation. Taken together, the above results demonstrate that the iucA and iucC genes, which participate in aerobactin synthesis, play an important role in the pathogenicity of the APEC strain E058.

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Author Contributions

Conceived and designed the experiments: SG XL. Performed the experiments: HP LX QG DZ YZ. Analyzed the data: JL. Contributed reagents/materials/analysis tools: SG. Wrote the paper: JL.

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