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The avian pathogenic *Escherichia coli* O2 strain E058 carrying the defined aerobactin-defective *iucD* or *iucDiutA* mutation is less virulent in the chicken

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ABSTRACT

The expression of aerobactin accounts for much of the pathogenesis of avian pathogenic *Escherichia coli* (APEC). *iucA*, *iucB*, *iucC* and *iucD* are involved in aerobactin synthesis and *iutA* is responsible for the expression of a specific outer membrane receptor protein for ferric aerobactin. Knockout mutants of *iucD* and *iucDiutA* in the APEC O2 strain E058 were constructed and named E058Δ*iucD* and E058Δ*iucD*Δ*iutA*, respectively. To evaluate the pathogenicity of these mutants, we used multiple approaches to assess the effects of mutations on the virulence of APEC E058. Aerobactin-defective mutants E058Δ*iucD* and E058Δ*iucD*Δ*iutA* showed significantly decreased pathogenicity compared with the wild-type strain E058, evidenced by the low extent of colonization in selected organs or being outcompeted by E058 *in vivo*. Chickens challenged with APEC E058 exhibited typical signs and lesions of avian colibacillosis, while those inoculated with the mutants E058Δ*iucD* or E058Δ*iucD*Δ*iutA* showed moderate airsacculitis, mild pericarditis and perihepatitis. However, no significant differences in resistance to specific-pathogen-free chicken serum, ingestion by HD-11 cells, and growth rates in LB were observed between the mutants and the wild-type strain. These results indicated that the IucD- and IutA-related virulence factors play a significant role in the pathogenesis of the APEC strain E058.

1. Introduction

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 disease of poultry, causing high morbidity and mortality in chickens and turkeys, thus leading to great economic losses (Russo and Johnson, 2000; Mokady et al., 2005). In immunocompromised hosts, APEC strains cause a variety of severe infections, including acute fatal septicemia, subacute pericarditis and airsacculitis caused entirely or partly by APEC.

Iron is a nutrient that is essential for metabolism in bacteria. The concentration of iron required for bacterial growth is 10^{-7} M, while in the normal body fluid of a vertebrate host is just 10^{-12} M. Invasive *E. coli* isolates harboring the ColV plasmid are able to cope with this restrictive free iron concentration (Waters and Crosa, 1991). ExPEC strains have acquired numerous iron transport systems to mediate iron uptake from the host (Garenaux et al., 2011). The ferrous iron transporters encoded by *feoAB* and *sitABCD* in commensal and pathogenic *E. coli* mediates iron import and contributes to APEC virulence (Kammler et al., 1993; Sabri et al., 2008). In addition, iron can be either directly scavenged from the extracellular space through haem transport systems such as ChuA (Torres et al., 2001) and Hma (Hagan and Mobley, 2009), or indirectly acquired via siderophore systems, low-molecular-weight chelators, including the catecholates enterobactin and salmochelin, the hydroxamate aerobactin, and yersiniabactin, a mixed-type siderophore (Henderson et al., 2009). While enterobactin is produced by nearly all *E. coli* strains, the genes encoding aerobactin, salmochelin and yersiniabactin receptors are found more frequently among pathogens (Carbonetti et al., 1986; Johnson and Stell, 2000; Kanamaru et al., 2003). The expression of aerobactin underlies much of the pathogenesis of APEC strains (Lafont et al., 1987). Compared with enterobactin, aerobactin is probably able to perform more efficiently as an iron acquisition system in serum (de Lorenzo and Martinez, 1988). Accordingly, it is regarded that aerobactin plays an independent role in the pathogenicity of APEC strains and persistent

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infections with them, especially in deep tissues (Dozois et al., 2003).

As a low molecular weight compound, aerobactin is encoded by five genes, all located at one operon. Among them, *iucA*, *iucB*, *iucC* and *iucD* participate in synthesizing the aerobactin, and the fifth, *iutA*, encodes the membrane receptor (Bindereif and Neilands, 1985; Carbonetti and Williams, 1984; Gross et al., 1984). The product of *iucA* is thought to be responsible for the first step in the synthetase reaction, *iucB* encodes N^{ϵ}-hydroxylysine acetyltransferase, *iucC* putatively encodes aerobactin synthetase and *iucD* is thought to encode N^{ϵ}-lysine monooxygenase (de Lorenzo et al., 1986). Although aerobactin genes were first found to be located on pColV plasmids (Warner et al., 1981), they have also been found to be chromosomally encoded in many instances (Lawlor and Payne, 1984; McDougall and Neilands, 1984; Bindereif and Neilands, 1985; Valvano et al., 1986; Marolda et al., 1987). When there is sufficient iron in the nutrient medium, the level of synthesis is reduced by 95%, whereas when chelating agents such as 2,2-dipyridy or nitrilotriacetate are added to the medium, iron levels become deficient and its synthesis is increased; this mechanism enables the bacteria to grow properly (Wayne et al., 1976).

APEC strains survive iron deprivation, particularly in the host, often by possessing iron acquisition systems and siderophores that compete with transferrins to scavenge iron (Dho and Lafont, 1984). Lafont et al. stated that aerobactin genes were present in APEC strains and absent from avirulent strains (Lafont et al., 1987). JanBen et al. showed that 133 out of 150 APEC strains tested (88.7%, 133/150) harbored *iucD* (JanBen et al., 2001). Delicato et al. (2003) collected two hundred APEC isolates from either the tracheal secreta or the livers of 200 chickens with clinical signs of colibacillosis and 50 isolates from the feces of healthy chickens. PCR was used to analyze these isolates, revealing that 63% of colibacillosis isolates possessed the *iutA* gene, while only 12% of isolates from healthy chickens carried the same gene ($p < 0.05$) (Delicato et al., 2003). Ewers et al. revealed that 71.1% of APEC isolates carried the *iucD* gene (Ewers et al., 2004). Someya et al. reported that all of 26 tested APEC isolates harbored the *iucD* gene (Someya et al., 2007).

To identify virulence-associated genes in APEC isolates, we applied selective capture of transcribed sequences (SCOTS) to screen putative virulence genes expressed *in vivo* in the APEC E058 strain (serotype O2). The *iutA* and *iucA* genes involved in the aerobactin system were selected (Chen et al., 2007). DNA microarrays were used to analyze the transcriptome of APEC E058 grown under both *in vivo* and *in vitro* conditions. Results in a chicken challenge model suggested that the *iutA* and *iucCD* genes were differentially upregulated in APEC E058, implying that these genes play a role in APEC E058 pathogenicity (Zhao et al., 2009). These findings suggested that genes coding for aerobactin may be potential virulence-associated genes with a role in the pathogenesis of APEC strains. To elucidate the roles of *iucD* and *iutA* genes in the pathogenesis of the APEC O2 strain E058, a series of pathogenicity tests including bactericidal assays of chicken serum, an HD-11 cell ingestion assay, assessment of the colonization of and persistence in chickens, and a competition experiment *in vivo*, were employed to evaluate the pathogenicity of APEC E058Δ*iucD* and E058Δ*iucD*-Δ*iutA* compared with the parent strain E058.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

All *E. coli* strains and plasmids used in this study are listed in Table 1. Oligonucleotide primers are listed in Table 2. All primers used for amplification of the genes were obtained from Sangon (Shanghai, China). Growth of bacterial cultures was performed at 37 °C in Luria–Bertani (LB) broth, on plates or within M9 medium. Mutant strains were selected on LB agar supplemented with zeocin at 25 μg/ml.

Growth rates of wild-type strain and mutants were measured as follows: an overnight culture was inoculated in 10 ml of LB medium (adjusted OD_{600nm} to 0.05) and shaken at 37 °C at 220 rpm. The bacterial cultures were monitored spectrophotometrically at OD_{600nm} for 4.5 h. The data shown represent the averages of three independent assays. *E. coli* was grown on LB agar plates or in LB broth with appropriate antibiotic supplementation. Antibiotics were added at the following concentrations: zeocin, 25 μg/ml; ampicillin, 60 μg/ml.

2.2. General DNA methods

DNA restriction endonucleases, the DNA Purification kit, the agarose Gel DNA Purification Kit, and DNA markers were purchased from Takara (Dalian, Liaoning, China). PCR amplification was carried out using 2× Taq Master Mix (Vazyme Biotech Co., ltd. Piscataway, NJ, USA). Purification of PCR products and DNA fragments was performed using kits manufactured by Promega (Madison, WI, USA). A High DIG DNA Labeling and Detection kit was purchased from Roche (Indianapolis, IN, USA). DNA nucleotide sequences were determined by Sangon (Shanghai, China) and analyzed using DNASTAR software (DNASTAR Inc., Madison, WI, USA).

2.3. Cloning of iucD and construction of an iucD mutant of E058

The λ Red recombinase system was used to construct the mutant E058Δ*iucD* (Datsenko and Wanner, 2000). Briefly, the plasmid pKD46 was electroporated into E058 to express Red recombinase. A boiled preparation of the overnight culture of E058 was used as template DNA for PCR amplification (Chen et al., 2007). The *iucD* gene was amplified from E058 template using the primers DF and DR (Fig. 1A, Table 2). The PCR product was gel purified following the manufacturer's instructions. The product was digested with *Sal*I-*Eco*RI, and then subcloned into the *Sal*I-*Eco*RI sites of pBluescript SKII(−) to form pS-*iucD*. To clone the Zeo^r gene into *iucD*, the *iucD* PCR product was amplified from pS-*iucD* using the primers SDF and SDR, the PCR product was digested with *Eco*RV-*Sph*I and the Zeo^r gene was obtained from pEM7/Zeo by *Eco*RV-*Sph*I digestion. Then, they were ligated to form pS-*iucD*-*Zeo*. After verification by sequence analysis, the disrupted *iucD* gene containing the inserted Zeo^r gene in pS-*iucD*-*Zeo* was amplified by PCR and purified for electroporation into E058 competent cells as described previously (Gao et al., 2012). After 24 h of incubation, the resulting Zeo^r colonies were selected for PCR identification using the primers DF and DR, and the inactivated *iucD* mutant was verified by sequencing.

2.4. Construction of iucDiutA double mutant of E058

Similarly, the λ Red recombinase system was used to construct mutant E058Δ*iucD*Δ*iutA*. The *iucDiutA* product was amplified from E058 template using the primers DAF and DAR (Fig. 1A, Table 2). The PCR product was gel purified following the manufacturer's instructions. The product was then subcloned into the TA-cloning vector pCR 2.1 to form pCR2.1-*iucDiutA*. The Zeo^r product was amplified from pEM7/Zeo using ZF and ZR primers (Table 2) and introduced into the *iucDiutA* genes at the internal *Nde*I-*Cla*I sites to form pCR2.1-*iucDiutA*-*Zeo*. After verification by sequence analysis, the disrupted *iucDiutA* gene containing the inserted Zeo^r gene was amplified by PCR and purified for electroporation into E058 competent cells. After 24 h of incubation, the resulting Zeo^r colonies were selected for PCR identification using the primers

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Table 1
Strains and plasmids used in this study.

	Description	Source
Strains		
E058	Wild-type avian *E. coli* serotype O2	Gao et al. (1999)
DH5α	endA1 hsdR17($r_K^- m_K^+$)supE44 thi-1 recA1 gyrA (Nal^R) RelA1Δ(lacIZYA-argF) U169deoR (ϕ80d lac Δ(lacZ) M15)	Invitrogen
E058Δ*iucD*	*iucD* mutant of E058, Zeo^R	This study
E058ΔiucDΔiutA	*iucDiutA* mutant of E058, Zeo^R	This study
ReE058Δ*iucD*	pGEX-6p-1-*iucD* complementation of E058Δ*iucD*, Amp^R	This study
Plasmids		
pKD46	Red recombinase helper plasmid, temp sensitive; Amp^r	Datsenko and Wanner (2000)
pGEM-T^REasyVector	TA cloning vector, Amp^R	Promega
pT-iucD	*iucD* cloned into pGEM-T^REasy Vector	This study
pBluescriptIISK(−)	Cloning vector, Amp^R	Fermentas
pS-iucD	*SalI-EcoRI iucD* fragment cloned into SK(−)	This study
pEM7/Zeo	Zeocin-resistant cassette	Invitrogen
pS-iucD-Zeo	Zeocin-resistant gene inserted into pS-*iucD*	This study
pCR 2.1	TA cloning vector, Amp^R	Invitrogen
pCR2.1-iucDiutA	*SalI-BamHI iucDiutA* fragment cloned into SK(−)	This study
pCR2.1-iucDiutA-Zeo	Zeocin-resistant gene inserted into pCR2.1-*iucDiutA*	This study
pGEX-6p-1	Expression vector	Amersham
pGEX-6p-1-*iucD*	*SalI-EcoRI iucD* fragment cloned into pGEX-6p-1	This study

Table 2
Primers designed and used in this study.

Primer code	Primer sequence 5′–3′	Positions (bp)	Source
DF	TCAGTCGACTCAGCATTGCTGCGTTGT *SalI*	4461–4478	This study
DR	CGCGAATTCTACGTGCAGATCTCCATG *EcoRI*	5592–5609	This study
SDF	GACGATATCTCATATGCTTCACACAGG *EcoRV*	4881–4898	This study
SDR	CCTGCATGCCTGGAGGAAGATATTCGC *SphI*	5141–5158	This study
DAF	CTCGTCGACGTTGTCACATCAGATCGG *SalI*	4474–4491	This study
DAR	CTCGGATCCTCAGGAATCAGGTAGTCC *BamHI*	7535–7552	This study
ZF	CTCCATATGCACGTGTTGACAATTAAT *NdeI*	1938–1955	This study
ZR	CTCATCGATTCAGTCCTGCTCCTCGGC *ClaI*	2366–2383	This study
RDF	CTCGAATTCATGAAAAAAAGTGTCGAT *EcoRI*	4412–4429	This study
RDR	CTCGTCGACTCAGCCCAAAGATGCTGT *SalI*	5723–5740	This study
Zeo-F	ATGGCCAAGTTGACCAGT	2009–2026	This study
Zeo-R	TCAGTCCTGCTCCTCGGC	2366–2383	This study
CF	GGGCTGGCTCTGGATTG	2837–2853	This study
CR	CATCGGGCTTTCAGTAGTT	2995–3013	This study
AF	TCAACCCACTGCTTCTTACC	5800–5819	This study
AR	GCCACGCACATTCATACC	6036–6053	This study
O2F	ATGTCGTGTTCCGTGCTCA	8097–8115	This study
O2R	TCAGTAAGTTGGCAGCATC	8300–8318	This study

DAF and DAR, and the inactivated *iucDiutA* mutant was verified by sequencing.

2.5. Complementation of the iucD mutant

To study complementation, the native *iucD* gene was obtained similarly using the primers RDF and RDR (Table 2) with the restriction enzyme recognition sites *Eco*RI and *Sal*I. To determine whether the sequence was in frame, the pGEM®-T Easy Vector with the *iucD* insert was sequenced by Sangon Co., Ltd. The *iucD* PCR product and expression vector pGEX-6p-1 were then digested using the restriction enzymes *Eco*RI and *Sal*I for 2 h at 37 °C, and ligated using T4 DNA ligase overnight at 4 °C. Three microliters of the ligation mix was then transformed into DH5α cells, which were plated out on LB agar containing ampicillin. Colonies were tested for the presence of *iucD* using the standard primers DF and DR (Table 2). The modified plasmid pGEX-6p-1 with the *iucD* insert was isolated from DH5α and electroporated into E058Δ*iucD* to complement the gene deleted.

2.6. Southern blotting

Genomic DNA was extracted from E058, E058Δ*iucD* and E058Δ*iucD*Δ*iutA* using the DNeasy Tissue Kit (Takara, Japan) following the manufacturer's instructions. The Zeo^r gene was amplified from pEM7/Zeo as a probe with the primers Zeo-F/Zeo-R (Table 2), which was DIG-labeled using the DIG High Prime DNA Labeling and Detection kit (Roche, Indianapolis, IN, USA). Genomic DNA samples were digested overnight at 37 °C using 100 units of the restriction enzymes *Eco*RI and *Hin*dIII (Takara, Japan), respectively. According to standard protocols, digested samples were electrophoresed overnight on 0.8% TBE agarose gel, transferred to a positively charged nylon membrane (Roche, USA) and fixed by baking at 120 °C for half an hour. After prehybridization in 10 ml DIG Easy Hyb work solution at 42 °C for 2–3 h, hybridization was carried out at 42 °C overnight in a hybridization oven. The membrane was washed twice in 2× SSC containing 0.1% SDS at room temperature for 5 min each, and then twice in 0.5% SSC containing 0.1% SDS at 65 °C for 15 min each. Genomic DNA hybridized with probe was immunologically detected by anti-DIG antibody conjugated with alkaline phosphatase (AP) (Roche, USA).

2.7. cDNA synthesis and RT-PCR

Total RNA was isolated from log-phase bacteria of each strain using an RNeasy Mini kit and treated with an on-column RNase-Free DNase set (Qiagen, Hilden, Germany). 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 the target genes *iucC*, *iucD*, *iutA* and *O2ColV16* in cDNA samples were CF/CR, DF/DR, AF/AR and O2F/O2R, respectively (Fig. 1A, Table 2). In parallel, PCRs were performed using plasmid DNAs as positive controls and RNA samples without activation of the reverse transcription reaction as negative controls. The PCR products were resolved on 0.8% agarose gels and visualized by ethidium bromide staining.

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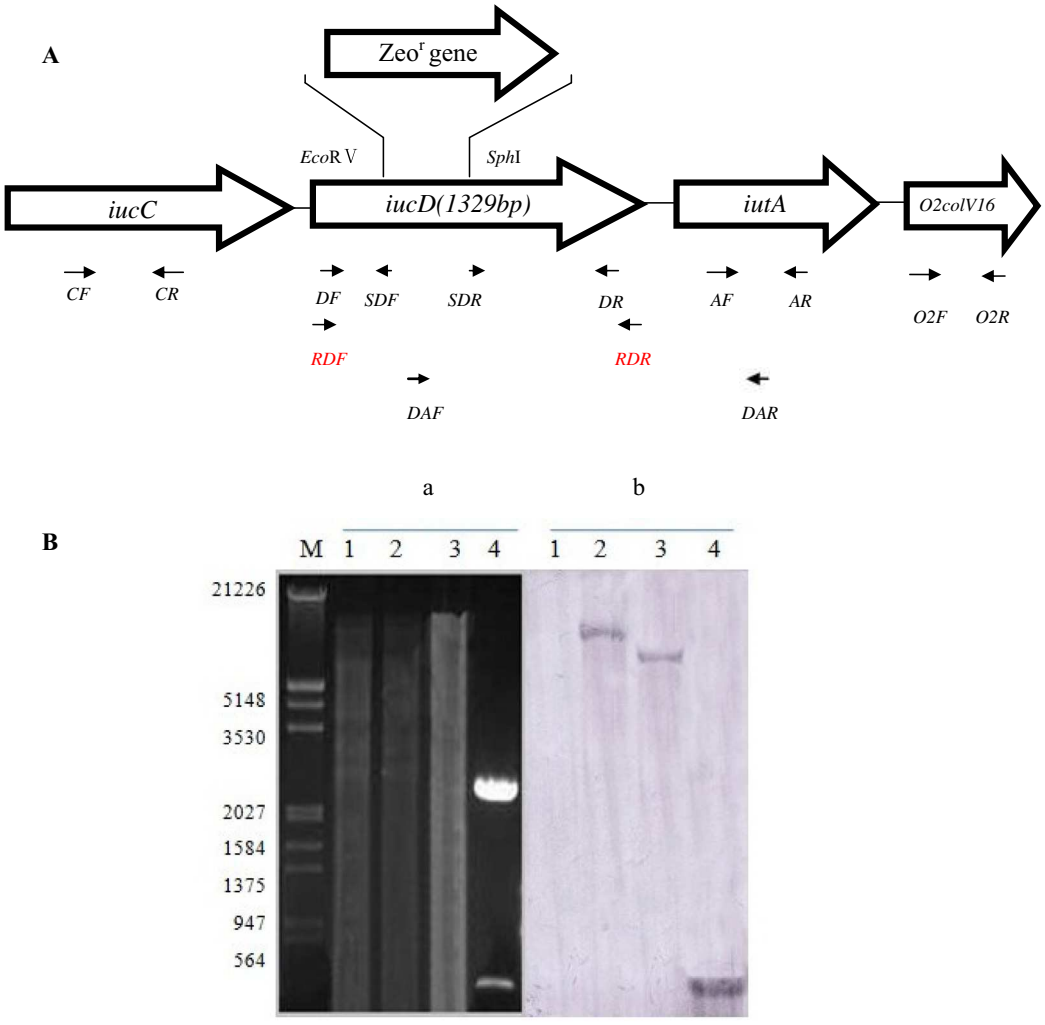

Fig. 1. (A) Genetic organization of the *iucD* and *iutA* loci in the APEC E058 pAPEC-O2-ColV-like plasmid DNA. The location of the deletion in *iucD* replaced by a Zeo^r gene is between *Eco*RV and *Sph*I cleavage sites introduced by PCR. A gene upstream of *iucD* encodes an aerobactin IucC protein (*iucC*), whereas a downstream gene encodes the aerobactin receptor IutA (*iutA*). The gene downstream of *iutA* is *O2ColV16*. Large arrows represent the direction of transcription, and the sites of primers used are indicated with small arrows. (B) Detection of the Zeo^r gene inserted into E058Δ*iucD* or E058Δ*iucD*Δ*iutA* pAPEC-O2-ColV-like plasmid DNA by Southern blotting. Lane 1, 2 and 3: plasmid DNA from E058, E058Δ*iucD* or E058Δ*iucD*Δ*iutA* plus *Eco*RI/*Hin*dIII. Lane 4: 3 μg of pEM7/Zeo (a plasmid with a Zeo^r cassette as positive control) plus *Eco*RI + *Xho*I. Each digested sample was resolved on a 0.7% agarose gel and visualized by ethidium bromide staining (a). Southern blotting was performed using a DIG-labeled Zeo^r (b). Lambda DNA *Eco*RI/*Hin*dIII molecular size standards (Takara) are shown in base pairs on the left (lane M).

2.8. Bactericidal assay with specific-pathogen-free chicken serum

Complement-sufficient specific-pathogen-free (SPF) chicken serum was prepared and pooled from ten SPF chickens (White Leghorn, Jinan SPAFAS poultry Co., Ltd., Jinan, Shandong, China). A bactericidal assay was performed in a 96-well plate (Peng et al., 2005). SPF chicken serum was diluted to 0.5%, 2.5%, 5.0%, 12.5% and 25.0% in pH 7.2 phosphate-buffered saline (PBS). Bacteria [10 μl containing 10^6 colony-forming units (CFU)] were inoculated into 190-μl reaction wells containing the diluted SPF chicken serum, 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.

2.9. HD-11 cell ingestion assay

For the ingestion assay, HD-11 cells (an avian macrophage cell line) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, New York, USA) containing 10% fetal bovine serum (FBS, PAA, Pasching, Australia) at 37 °C in 5% CO_2 with 2×10^5 cells per well in 24-well cell culture plates, and incubated for 24 h prior to ingestion assays. Bacteria were inoculated into cells with a multiplicity of infection (M.O.I.) of 100. Inoculated cells were incubated at 37 °C for 1 h under 5% CO_2 to allow the bacteria to invade into the cells. After that, the medium was washed using PBS. Then, the extracellular bacteria were eliminated by DMEM medium containing gentamicin (100 μg/ml) at 37 °C for 1.5 h prior to washing cells using PBS, and the intracellular bacteria were treated with 1 ml of 0.1% Triton X-100. A 100-μl aliquot of the resulting suspension was inoculated into 900 μl of PBS. Serial dilutions (1:10) of each well 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 bacterial cells by the initial inoculation bacterial number.

2.10. Colonization and persistence of the mutants

Animals were infected with the wild-type strain, mutants and the complementation strain to determine their colonization and

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persistence abilities during systemic infection. 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, 3-week-old SPF chickens (White Leghorn, Jinan SPAFAS poultry Co., Ltd.) were inoculated via the left thoracic air sac with a bacterial suspension containing 10^7 CFU. 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 the isolated bacteria were determined by plating serial dilutions of the homogenates onto LB agar plates with or without zeocin.

2.11. In vivo competition tests

For *in vivo* competition assays, 3-week-old white leghorn SPF chickens were inoculated with cultures of the wild-type strain E058 and its mutant E058Δ*iucD* or E058Δ*iucD*Δ*iutA* mixed in a ratio of 1:1 (1×10^7 CFU for each strain) via the left air sac. Chickens were provided with food and water *ad libitum*. Twenty-four hours after infection, the hearts, livers, spleens, lungs and kidneys of inoculated birds were collected, weighed and homogenized, and serial dilutions were plated on LB medium with or without antibiotics for selection of mutants or total bacteria, respectively. A competitive index (CI) was calculated for the mutant by dividing the output ratio (mutant/wild type) by the input ratio (mutant/wild type). Based on this CI, mutants that outcompeted the wild-type strain by up to 10-fold were evaluated as slightly attenuated, those that outcompeted up to 100-fold were evaluated as moderately attenuated, and those that outcompeted by >100-fold were evaluated as highly attenuated (Li et al., 2005).

2.12. Aerobactin production

Mutants were also assessed for aerobactin production as described by Vidotto et al. (1990). Low-iron agar assay plates, composed of M-9 minimum salts, containing 200 μM 2,2′-dipyridyl and 0.2% glucose, were seeded with 1 ml/l 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, its mutants and the complementation strain 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

2.13. Statistical analyses

Statistical analyses for *in vivo* tests were performed using the GraphPad Prism v5.0 software package (GraphPad Software, USA). For the competition assay, the Wilcoxon matched-pair test was performed. For the colonization and persistence assay, the Mann–Whitney test was performed.

3. Results

3.1. Genetic analysis of the iucD sequence

Based on the nucleotide sequence of *iucD* in the plasmid pAPEC-O2-ColV of the *E. coli* strain A2363 (accession no. AY553855), the PCR primers DF/DR were designed to amplify the *iucD* gene. Analysis of the promoter and open reading frame (ORF) of APEC E058 showed that the *iucD* DNA fragment contained an ORF of 1329 bp with a predicted gene product of 442 amino acids. The deduced amino acid sequence of *iucD* of APEC E058 showed 100% identity to that of the APEC strain A2363. Sequence analyses revealed that the gene upstream of *iucD* is *iucC*, encoding an aerobactin IucC protein. The 3′ end of *iucD* showed a 3-bp overlap with the 5′ end of *iucC*. The gene immediately downstream of *iucD* is *iutA*, encoding an aerobactin receptor, IutA. Between *iucD* and *iutA*, a 25 bp non-coding region is present, which does not contain promoter and regulator elements for *iucD* (Fig. 1A).

3.2. Construction and characterization of iucD and iucDiutA knockout mutants

Nucleotide sequence analysis of PCR products confirmed that the *Zeo* cassette had been inserted into the pAPEC-O2-ColV-like plasmid at the predicted position. The mutants were named E058Δ*iucD* and E058Δ*iucD*Δ*iutA*.

Southern blotting was performed to determine whether a single copy of the Zeo^r gene was inserted into the pAPEC-O2-ColV-like plasmid DNA. The parental E058, E058Δ*iucD* and E058Δ*iucD*Δ*iutA* plasmid DNAs were digested with *Eco*RI/*Hin*dIII (Fig. 1Ba) and probed with digoxigenin (DIG)-labeled Zeo^r gene (Fig. 1Bb). Only one band was detected in the plasmid DNA of E058Δ*iucD* (Fig. 1Bb, lane 2) or E058Δ*iucD*Δ*iutA* (Fig. 1Bb, lane 3), but none was detected in that of E058 (Fig. 1Bb, lane 1), showing that there was a single Zeo^r gene insertion in the pAPEC-O2-ColV-like DNA of the mutants.

To determine whether the insertion had a polar effect on the upstream or downstream gene, total RNA isolated from E058, E058Δ*iucD* or E058Δ*iucD*Δ*iutA* was subjected to RT-PCR analysis using primer sets designed for *iucC* (CF/CR), *iucD* (DF/DR), *iutA* (AF/AR) and *O2ColV16* (O2F/O2R). Compared with E058, insertion of the Zeo^r gene in E058Δ*iucD* only disrupted *iucD* gene transcription (Fig. 2A, lane 10), while in E058Δ*iucD*Δ*iutA*, both *iucD* and *iutA* genes were disrupted (Fig. 2B, lane 12, 14).

3.3. Aerobactin production

In testing for aerobactin production, the wild-type strain E058 and the complementation strain Re-E058Δ*iucD* showed the ability to produce aerobactin, while the E058Δ*iucD*, E058Δ*iucD*Δ*iutA* mutants lost the ability to produce aerobactin (Fig. 3).

3.4. Morphology and growth rates of E058ΔiucD and E058ΔiucDΔiutA mutants

Compared with the parental strain, the colonies of isogenic mutants 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 (Fig. 4).

3.5. Bactericidal effect of SPF chicken serum on E058 and isogenic mutants

In a bactericidal assay, the abilities of mutant E058Δ*iucD* and E058Δ*iucD*Δ*iutA* to survive in SPF chicken serum was not affected (Fig. 5), indicating that the aerobactin system may be unrelated to serum complement resistance.

3.6. Ingestion of APEC E058 and its mutants by the HD-11 cell line

The results obtained using HD-11 to study bacterial ingestion showed no significant differences between the isogenic mutants and the parent strain E058. The ingestion ratios of HD-11 to E058, E058Δ*iucD* and E058Δ*iucD*Δ*iutA* were 0.23%, 0.21% and 0.19% ($p > 0.05$), respectively. Thus, we assumed that the deletion of either the single gene *iucD* or the two genes *iucD* and *iutA* did not influence the ingestion of APEC E058 by HD-11 cells.

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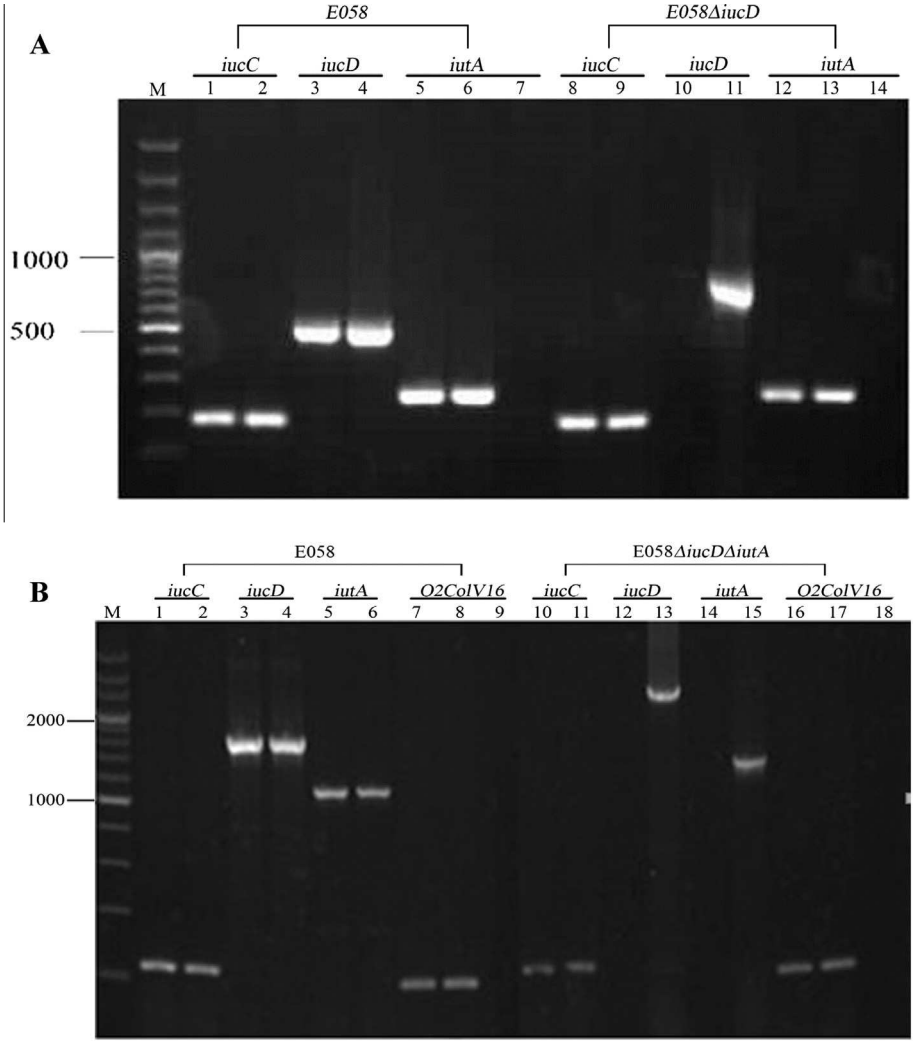

Fig. 2. Detection of target gene transcription by RT-PCR. (A) Detection of target genes transcription in E058 and E058Δ*iucD* by RT-PCR. Templates: lanes 1, 3, 5: cDNA derived from total RNA of E058. Lanes 2, 4, 6: plasmid DNA from E058. Lane 7: total RNA from E058 without activation of RT. Lanes 8, 10, 12: cDNA derived from total RNA of E058Δ*iucD*. Lanes 9, 11, 13: plasmid DNA from E058Δ*iucD*. Lane 14: total RNA from E058Δ*iucD* without activation of RT. Target genes: Lanes 1, 2, 8, 9: *iucC*; Lanes 3, 4, 10, 11: *iucD*; Lanes 5, 6, 7, 12, 13, 14: *iutA*. (B) Detection of target genes transcription in E058 and E058Δ*iucD*Δ*iutA* by RT-PCR. Templates: lanes 1, 3, 5, 7: cDNA derived from total RNA of E058. Lanes 2, 4, 6, 8: plasmid DNA from E058. Lane 9: total RNA from E058 without activation of RT. Lanes 10, 12, 14, 16: cDNA derived from total RNA of E058Δ*iucD*Δ*iutA*. Lanes 11, 13, 15, 17: plasmid DNA from E058Δ*iucD*Δ*iutA*. Lane 18: total RNA from E058Δ*iucD*Δ*iutA* without activation of RT. Target genes: Lanes 1, 2, 10, 11: *iucC*; Lanes 3, 4, 12, 13: *iucD*; Lanes 5, 6, 14, 15: *iutA*; Lanes 7, 8, 9, 16, 17, 18: *O2ColV16*.

3.7. The colonization and persistence of the mutants

By determining colony numbers in selected organs at 24 h post-inoculation, the capacity of colonization and persistence of the wild-type strain and its mutants to colonize internal tissues was assessed. At 24 h after infection, the wild-type strain E058 colonizing internal tissues with maximum loads was observed in the lung (6.8×10^5 CFU g^{-1}) as well as in the spleen (4.2×10^5 CFU g^{-1}); minimal colony counts were obtained in heart (8.2×10^3 CFU g^{-1}), while the colony numbers in liver (2.6×10^4 CFU g^{-1}) and kidney (2.2×10^4 CFU g^{-1}) were intermediate for the parent strain E058 (Fig. 6). Compared with the wild-type strain E058, the mutant E058Δ*iucD* showed significantly reduced bacterial numbers in lung (1.2×10^3 CFU g^{-1}) ($p < 0.01$), spleen (2.5×10^3 CFU g^{-1}) ($p < 0.01$), heart (78 CFU g^{-1}) ($p < 0.01$), liver (1.1×10^2 CFU g^{-1}) ($p < 0.01$) and kidney (1.2×10^2 CFU g^{-1}) ($p < 0.01$), while the mutant E058Δ*iucD*Δ*iutA* also demonstrated significantly reduced numbers in all selected organs, with especially low numbers in lung (7.6×10^2 CFU g^{-1}) ($p < 0.01$), spleen (1.8×10^3 CFU g^{-1}) ($p < 0.01$), heart (67 CFU g^{-1}) ($p < 0.01$), liver (3.2×10^2 CFU g^{-1}) ($p < 0.01$), kidney (1.1×10^2 CFU g^{-1}) ($p < 0.01$) (Fig. 6). On average the mutant strains were reisolated at a level 10–1000 times lower than the wild-type strain from the tested internal organs at 24 h post-challenge. The E058Δ*iucD* complementation strain restored the virulence and colonized internal organs of inoculated birds to the same extent as the wild-type strain ($p > 0.05$, Fig. 6). Chickens that were challenged with wild-type strain E058 developed moderate bilateral airsacculitis, congestion of the spleen, and moderate pericarditis. In contrast, the mutant E058Δ*iucD* caused only mild pericarditis and pulmonary congestion, while E058Δ*iucD*Δ*iutA* caused no conspicuous changes in the tissues tested.

3.8. Competition between the APEC wild-type strain E058 and its mutants in vivo

The capacities of isogenic mutants to compete for growth in chicken tissues with wild-type strain E058 were compared by *in vivo* competition assay. At 24 h post-challenge, the E058Δ*iucD*

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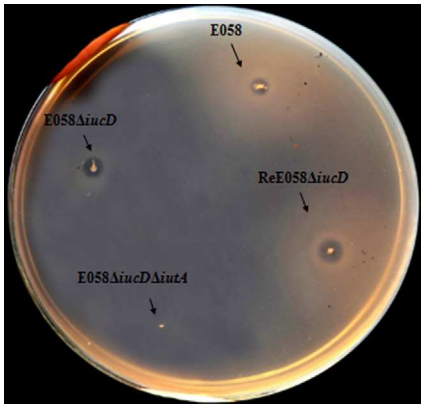

Fig. 3. Aerobactin production of mutants were determined by cross-feeding *E. coli* LG1522. Low-iron agar assay plates, composed of M-9 minimum salts, containing 200 μM 2,2′-dipyridyl and 0.2% glucose, were seeded with 1 ml/l of an overnight culture of the indicator organism, *E. coli* LG1522. The E058 strain, its mutants and the complementation strain were stab inoculated into the agar, and the plates were incubated at 37 °C for 24 h. Growth of the indicator organism around the stabs in a halo indicated aerobactin production by the test strains.

mutant showed slightly attenuated growth in all selected organs ($p > 0.05$) (Fig. 7A), with mean competitive indexes (CIs) of 0.125, 0.118, 0.135, 0.122 and 0.141 in the hearts, livers, spleens, lungs and kidneys of challenged birds, respectively. Growth of E058Δ*iucD*Δ*iutA* was highly attenuated in all five tissues ($p < 0.01$) (Fig. 7B), with the mean CIs of 0.0095, 0.0082, 0.0079, 0.0064 and 0.0074 in the hearts, livers, spleens, lungs and kidneys, respectively.

4. Discussion

The pathogenicity of APEC is known to require a variety of virulence factors and the mechanism by which APEC exerts pathogenesis need to be further investigated. Like most bacteria, APEC must acquire iron to survive, since iron is a nutrient that is essential for metabolism in bacteria. Therefore, APEC strains attempting to establish an infection must have the ability to scavenge iron and multiply within the iron-limited host environment. The hydroxamate aerobactin belonging to siderophore systems is a high-affinity iron-chelator and frequently produced by extra-intestinal *E. coli* which cause various types of infection (mostly, that of *E. coli* strains causing septicemia and urinary tract infections). Furthermore, the role of iron acquisition by aerobactin in virulence has already been well investigated in other pathogens (Nassif and Sansonetti, 1986; Nassif et al., 1987; Johnson et al., 1988).

Although the aerobactin biosynthesis genes are more frequently detected in pathogenic *E. coli* strains, they have also been found to be present in commensal strains based on the molecular epidemiological studies (Delicato et al., 2003). Moreover, since iron acquisition systems are assumed to be functionally redundant (Garcia et al., 2011; Landgraf et al., 2012), the role of aerobaction in the virulence of APEC need further investigation. As is known to all, the aerobactin operon is consisted of four biosynthesis genes *iucABCD* and one receptor gene *iutA*, However, prior to our studies, aside from the whole *iucABCDiutA* operon or the single *iutA* aerobactin receptor, the individual roles of *iuc*-containing genes in the virulence of APEC have seldom been investigated.

Previous studies for evaluating of the role of aerobaction in the virulence of APEC were often conducted by deleting the whole aerobactin operon, however, the results got by different research

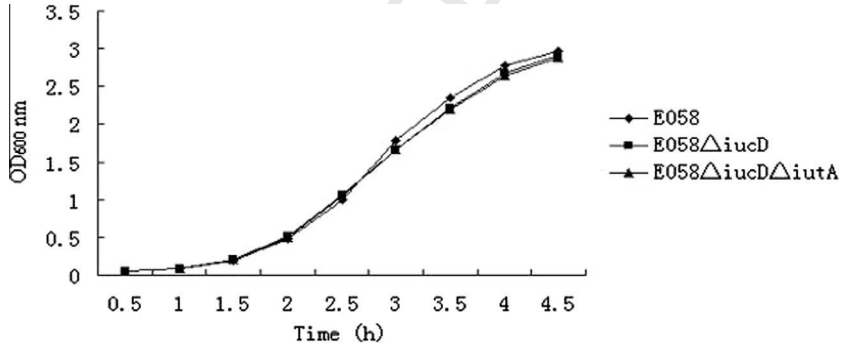

Fig. 4. Growth curves of E058 (♦), E058Δ*iucD* (■) and E058Δ*iucD*Δ*iutA* (▲) strains in LB broth at 37 °C. Optical density was checked every 0.5 h for 4.5 h. The data represent the averages of three independent assays.

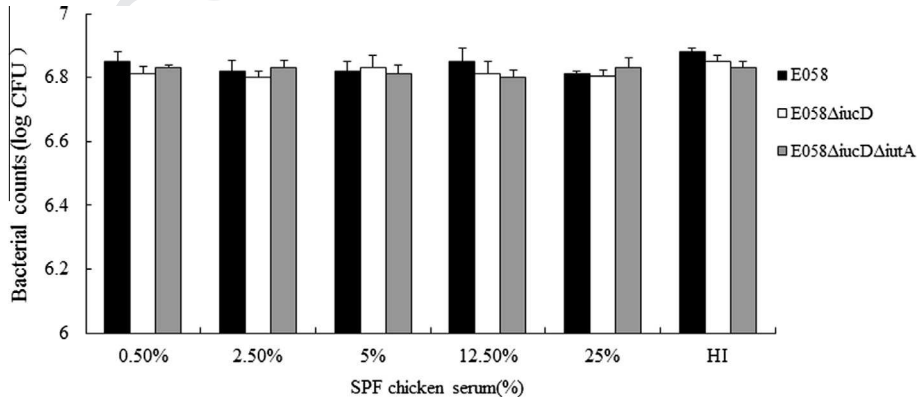

Fig. 5. Bactericidal activity of SPF chicken serum against the wild type strain and mutants. Strains E058 (black bar), E058Δ*iucD* (white bar), E058Δ*iucD*Δ*iutA* (gray bar). HI represents the group of heat-inactivated 25% SPF chicken serum used as controls for each strain tested. Data represent averages of three independent assays.

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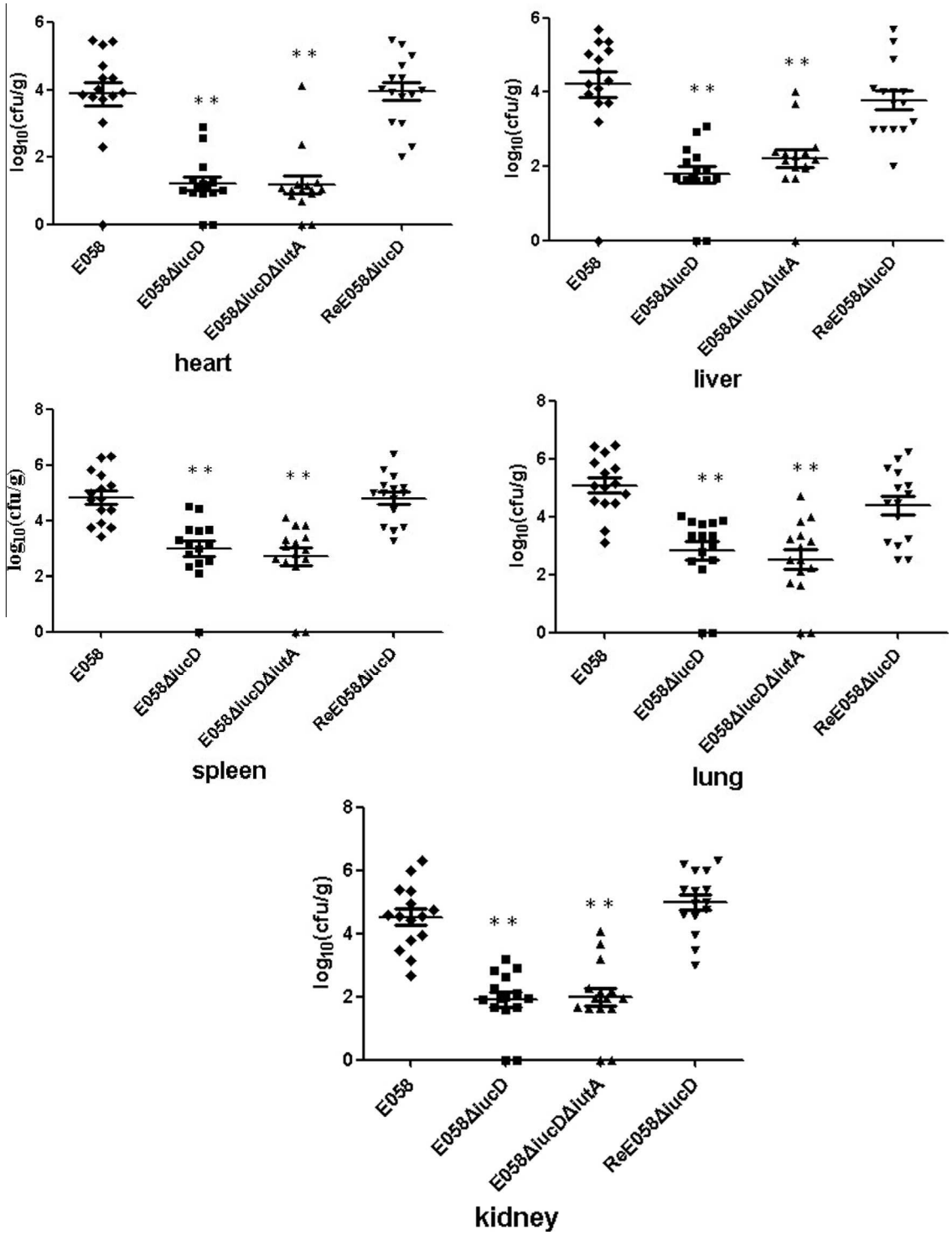

Fig. 6. Colonization and persistence of the wild-type strain E058 (♦), E058Δ*iucD* complementation (▼), mutants E058Δ*iucD* (■) and E058Δ*iucD*Δ*iutA* (▲) during systemic infection. Data are presented as $\log_{10}$ CFU g^{-1} of tissues. Horizontal bars indicated the mean $\log_{10}$ CFU g^{-1} values. Each data point represented a sample from an individual chicken. Statistically significances as determined by the Mann–Whitney test are indicated by asterisks (**$P < 0.01$).

groups were contradictory to some extent, which make it a little confusing. One group represented by Dozois et al. stated that an APEC mutant (derived from strain χ7122; serogroup O78) in which *iucABCDiutA* and *tsh* (encoding a temperature-sensitive hemagglutinin) are deleted was attenuated, because the bacterial numbers were significantly reduced in the lungs, livers, and spleens of chickens infected with the Aerobactin, Tsh mutant (χ7301) compared with the wild-type parent strain (χ7122) 48 h after infection (Dozois et al., 2003). Caza et al. investigated the relative roles of salmochelins and aerobactin in APEC virulence, and described an aerobactin and salmochelin mutant that was completely attenuated *in vivo*. They also demonstrated that aerobactin was produced at high rates *in vivo* at sites of infection (Caza et al., 2008). However, the other group represented by Skyberg et al. found that mutant APEC O2 Δ*vir2* with both the *sit* (encoding *sit ABC* transport system) and aerobactin operon deleted from the plasmid

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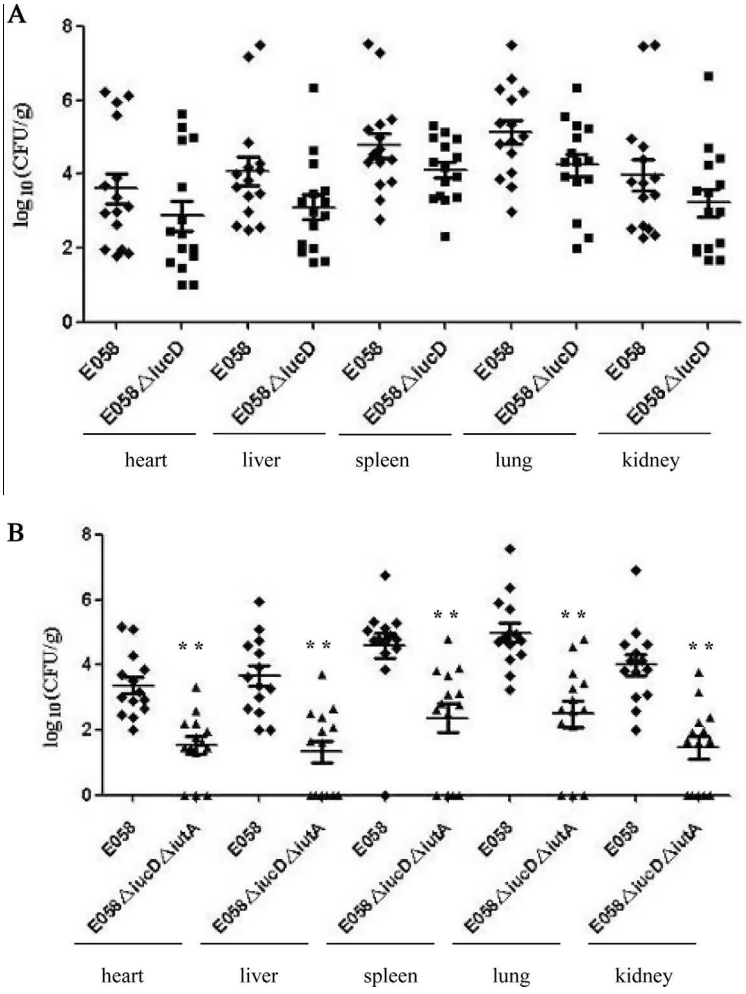

Fig. 7. *In vivo* competition assays. (A) E058 (♦) and mutant E058Δ*iucD* (■) were inoculated simultaneously. (B) E058 (♦) and E058Δ*iucD*Δ*iutA* (▲) were inoculated simultaneously. Data are presented as $\log_{10}$ CFU g^{-1} of tissues. Horizontal bars indicate the mean $\log_{10}$ CFU g^{-1} values. Each data point represents a sample from an individual chicken. Statistically significant differences in values between E058 and its mutants are indicated with asterisks ($^{**}P < 0.01$).

pAPEC-O2-ColV of the APEC O2 strain, showed no significant differences in lethality to chick embryos and growth in human urine compared with the parental strain APEC O2 (Skyberg et al., 2008). The contradiction between these results might be due to the differences in the strains (O78 vs O2) or the infection model (3 weeks-old chicken infection model vs chicken embryos lethality assay). Since all of them deleted not only the whole aerobactin operon (*iucABCDiutA*) but also along with the defined virulence genes *tsh* or *iro* (Dozois et al., 2000; Caza et al., 2008), the extent to which aerobactin affect the pathogenicity of APEC isolates was not clearly identified. To evaluate the role of aerobactin in virulence of APEC, we used an APEC strain E058 (O2) as a model strain to assess the specific importance of individual *iuc* genes for APEC virulence. In a former study from our lab, we constructed the *iucB* and *iucBiutA* mutants. We adopted both the the 3-week-old chicken infection model and chicken embryos lethality assay to analyze the roles of these two genes in APEC infections. Our results showed that the experimental infection of 3-week-old chickens with the isogenic mutants led to significant reduction of bacterial loads in internal organs, but the mutants did not show any attenuation in the lethality to chick embryos compared to the wild-type strain (Xiong et al., 2012). As indicated by Skyberg, the embryo lethality assay may lack the sensitivity to detect changes owed to the mutated genes, since none of the isogenic mutants they created were attenuated in this model, including the defined virulence genes *tsh* and *iroN* (Skyberg et al., 2008). Furthermore, we have studied the effects of the defective mutations of the other *iuc* genes (*iucA*, *iucC* and the ferric aerobactin receptor *iutA*) and demonstrated their independent roles in the pathogenesis of APEC (Ling et al., 2013).

To date, the roles of single *iucD* gene and double *iucDiutA* genes in the pathogenesis of APEC strains have seldom been researched, so it will be valuable to elucidate these clearly. In this study, the mutants E058Δ*iucD* and E058Δ*iucD*Δ*iutA* were constructed and characterized to reveal an independent role or potentially synergistic effect between *iucD* and *iutA* genes on the pathogenicity of APEC O2 strain E058. In LB broth, there was no obvious difference in the growth of the mutants and wild-type strain. The similarity of growth rates between isogenic mutants and the wild-type strain indicated that the attenuation observed was not due to a general growth defect. During the assays of bacterial resistance to SPF chicken serum or ingestion by HD-11 cells, no obvious difference was observed between the wild-type E058 and its mutants. In this study, both mutants showed significantly decreased colonization compared with the wild-type strain in all organs tested in the single-strain challenge model, which were in accordance with our previous reports associated with the other *iuc* genes, implying that the overall *iuc* biosynthesis genes of aerobactin play critical role in the virulence of APEC E058 in chickens. The capacity of the E058Δ*iucD* and E058Δ*iucD*Δ*iutA* mutants to compete for growth

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in 3-week-old SPF chicken tissues with the wild-type strain E058 were also evaluated in a co-infection model. However, in the co-infection model, the *iucD* mutant was less attenuated than the *iucDiutA* mutant compared with the wild-type strain, with bacterial loads slightly reduced compared with E058 ($p > 0.05$). This was probably because the mutant is still able to import aerobactin produced by the wild-type strain in the coinfection model. This result was somewhat similar to the observation of Torres et al. (2001), who described that an *iucBentD* double mutant, defective for synthesis of both siderophores, was rescued by coinfection with a wild-type strain in the mouse UTI model. Their results implied that the exogenous siderophores synthesized by the wild-type strain may be sufficient to suppress the effects of the siderophore synthesis mutations in the coinfection model (Torres et al., 2001). Taken together, these results suggest that the single *iucD* gene mediating aerobactin biosynthesis is likely to be involved directly or indirectly in iron uptake to mediate the pathogenicity of APEC E058, and that there is a synergistic effect between *iucD* and *iutA* genes on the pathogenicity of APEC E058.

5. Conclusions

We investigated the extent to which *iucD* and *iutA*-related virulence factors affect the pathogenicity of APEC isolates. *iucD* and *iutA*-related virulence factors were shown to play a significant role in the pathogenesis of the APEC E058 strain. The exact mechanism underlying this role needs further study.

Uncited references

Baumler et al. (1998) and Russo et al. (1999).

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