Escherichia coli type III secretion system 2 regulator EtrA promotes virulence of avian pathogenic Escherichia coli

Shaohui Wang,¹ Xuan Xu,¹,² Xin Liu,¹ Dong Wang,¹ Hua Liang,¹ Xiaojun Wu,¹ Mingxing Tian,¹ Chan Ding,¹ Guijun Wang² and Shengqing Yu¹,*

Abstract

The Escherichia coli type III secretion system 2 (ETT2) is found in most E. coli strains, including pathogenic and commensal strains. Although many ETT2 gene clusters carry multiple genetic mutations or deletions, ETT2 is known to be involved in bacterial virulence. In enterohaemorrhagic E. coli (EHEC), ETT2 affects adhesion through the regulator EtrA, which regulates transcription and secretion of the type III secretion system (T3SS) encoded by the locus of enterocyte effacement (LEE). To date, no studies have been conducted on the role of EtrA in the virulence of avian pathogenic E. coli (APEC), which harbours only ETT2. Thus, we constructed etrA mutant and complemented strains of APEC and evaluated their phenotypes and pathogenicities. We found that the etrA gene deletion significantly reduced bacterial survival in macrophages, and proliferation and virulence in ducks. In addition, the etrA gene deletion reduced expression of the APEC fimbriae genes. Upregulation of genes encoding the pro-inflammatory cytokines interleukin (IL)-1β and IL-8 was also observed in HD-11 macrophages infected with the etrA gene mutant strain compared to the wild-type strain. Furthermore, the altered capacities of the mutant strain were restored by genetic complementation. Our observations demonstrate that the ETT2 regulator EtrA contributes to the virulence of APEC.

INTRODUCTION

Type III secretion systems (T3SSs) are widely used by Gram-negative bacteria to deliver effector proteins into eukaryotic cells in order to subvert host cellular processes and facilitate bacterial growth, survival and virulence [1–4]. Some bacterial pathogens employ multiple T3SSs for different pathogenic processes [5, 6]. In Escherichia coli, two distinct T3SSs have been identified and characterized. The locus of enterocyte effacement (LEE), which encodes T3SS in intestinal E. coli such as enteropathogenic E. coli (EPEC) and enterohaemorrhagic E. coli (EHEC), is essential for the formation of attaching and effacing (A/E) lesions [7]. A second T3SS, the E. coli type III secretion system 2 (ETT2), is more prevalent in intestinal E. coli strain [8–14].

Although ETT2 is thought to be a non-functional T3SS due to multiple genetic mutations and deletions, ETT2 has been shown to be involved in bacterial virulence [10, 15]. Several transcriptional regulators located in the ETT2 cluster, including YgeH, Yqel, YgeK/EtrB, EtrA and EivF, have been shown to regulate the expression of virulence genes of intestinal pathogenic E. coli. For example, EtrA and EivF from EHEC O157 were shown to have negative effects on LEE expression and adherence to epithelial cells [16]. In contrast, the regulator YgeK/EtrB can activate LEE expression and promote A/E lesion formation by directly interacting with the ler regulatory region [17]. Although regulator YgeH is required for expression of ETT2 genes, it appears to be non-functional for virulence gene regulation in enteroaggregative E. coli (EAEC) strain 042 [18]. Finally, another putative regulator, Yqel, has not been characterized.

ETT2 is crucial for the invasion, intracellular survival and virulence of extraintestinal pathogenic E. coli (ExPEC), including avian pathogenic E. coli (APEC) and newborn meningitis E. coli (NMEC) [19–21]. APEC is of particular concern, as it can cause serious extra-intestinal diseases in poultry, including air sacculitis, pneumonia and septicaemia, leading to devastating economic impacts on the poultry industry.
industry and potential risks to human health [22–25]. Our previous study verified that ETT2 is widely distributed among APEC strains [9]. However, the function of ETT2 regulators in APEC remains poorly understood. Therefore, this study was performed to determine the effects of the regulator EtrA on the phenotype and virulence of APEC. Our findings indicate that EtrA is involved in APEC virulence through regulation of transcription of the virulence factors.

**METHODS**

**Bacterial strains, plasmids and growth conditions**

The bacterial strains and plasmids used in this study are listed in Table 1. APCE94 is a serotype O78 APEC strain that was isolated from a chicken with clinical colibacillosis symptoms in Jiangsu, China. Our previous infection studies confirmed that this strain can cause severe colibacillosis symptoms and high mortality rates in chickens and ducks [9]. Furthermore, APCE94 was shown by PCR analysis to harbour several virulence genes, including fimC, tsh, mat, ompA, aatA, fyuA, irp2, iroN, iss, neuC, ibeB, yijp and cva/cvi. In addition, ducks immunized with an inactivated APCE94 vaccine are resistant to infection with different virulent APEC O78 strains (data not shown). Thus, strain APCE94 was used for infection studies, mutant strain construction and functional assays in this study [9, 21]. All E. coli strains were grown in Luria–Bertani (LB) medium at 37 °C with aeration. When necessary, LB medium was supplemented with ampicillin (100 µg ml⁻¹) or chloramphenicol (30 µg ml⁻¹). Plasmid DNA was isolated using the High Pure Plasmid Miniprep Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s guidelines. DNA manipulations and transformations were performed using standard methods.

**Sequence analysis**

The promoter of the etrA gene was predicted using prediction program tools available at http://www.fruitfly.org/seq_tools/promoter.html. Analyses of predicted protein motifs, structure and homology domains were performed using MOTIF Search, Phyre2 server tools and the BLASTP program from NCBI.

**Construction of mutant and complemented strains**

The etrA deletion mutant strain was generated using the lambda Red recombinase system as described previously [26]. Briefly, linear PCR products were transformed into APCE94 carrying the pKD46 plasmid and the etrA gene was replaced with a chloramphenicol resistance cassette. The chloramphenicol resistance cassette was then eliminated by the helper plasmid pCP20 and chloramphenicol-sensitive mutant strains were selected. The mutant strain was confirmed by PCR and sequencing, and designated as APCE94ΔetrA. For generation of complemented strains, the etrA gene, including its putative promoters, was amplified and subcloned into plasmid pSTV28 using the primer pair etrACo-F and etrACo-R (Table 2). The recombinant plasmid pSTV28-etrA was then transformed into the mutant strain APCE94ΔetrA to construct the complemented strain APCE94CΔetrA.

**Growth curve and motility assays**

To determine whether etrA gene deletion affects bacterial growth and motility, the growth kinetics and motility haloes of strains APCE94, APCE94ΔetrA and APCE94CΔetrA were determined on LB medium and plates as described previously [21, 27]. Briefly, bacteria were incubated at 37 °C with shaking and the optical density of each strain was monitored at 1 h intervals for 16 h by spectrophotometry (Bio-Rad, Hercules, CA, USA). Bacterial motility haloes on LB soft agar motility plates (0.5 % agar) were measured after incubation at 37 °C for 12 h.

**Serum bactericidal assay**

Bacterial resistance to serum was determined as described previously, with some modifications [21, 27]. Briefly, bacteria were incubated with specific-pathogen-free (SPF) chicken serum at dilutions of 12.5, 25 and 50 % at 37 °C for 30 min. The surviving bacteria were counted after plating on LB agar plates. Heat-inactivated serum was used as a negative control. The survival rate was normalized to the number of bacteria treated with heat-inactivated serum.

### Table 1. Bacterial strains and plasmids used in this study

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<th>Strains or plasmids</th>
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Bacterial adherence and invasion assays

Bacterial adherence and invasion assays were performed as described previously [21, 27, 28]. Briefly, bacteria were grown to the logarithmic phase, pelleted and washed three times with Dulbecco’s modified Eagle’s medium (DMEM). Chicken embryo fibroblast DF-1 cell monolayers were washed and incubated with bacteria at an MOI of 100 for 2 h at 37°C and 5% CO₂. After washing with PBS to remove non-adherent bacteria, cells were lysed with 0.5% Triton X-100 and spread onto LB agar plates for counting. For invasion assays, cell culture, bacterial infection and bacterial counting were performed as described for the bacterial adherence assay. The infected cells were washed and treated with DMEM containing gentamicin (100 µg ml⁻¹) for 1 h to kill extracellular bacteria. Cells were then washed and lysed with 0.5% Triton X-100, and invasive bacteria were counted after plating on LB agar plates. All assays were performed three times in triplicate.

Intracellular survival assays

To determine the survival capacity of bacteria in macrophages, an intracellular survival assay was performed as described previously [21, 27]. Briefly, avian macrophage HD-11 cells were infected with bacteria as described for invasion assays. At 1 h post-infection (p.i.), cells were washed and treated with DMEM containing gentamicin (100 µg ml⁻¹) for 1 h to kill extracellular bacteria. To determine intracellular survival, cells were grown in DMEM containing 10 µg ml⁻¹ gentamicin for an additional 6, 12 or 24 h, washed and lysed before bacterial counting. The intracellular survival ratio was expressed as the change (n-fold) in the number of bacteria at a given time point relative to the initial number of invasive bacteria.

Ethical approval

All animal experiments were conducted in strict accordance with the Guiding Principles for the Humane Treatment of Laboratory Animals, as issued by the Ministry of Science and Technology of the People’s Republic of China (Policy No. 2006 398). The experiments described in this study were approved by the Institutional Animal Care and Use Committee at the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences (permit No: Shvri-Po-0244). One-day-old Cherry Valley ducks were purchased from Zhuanghang duck farm (Shanghai, China) and kept under a controlled temperature (28–30°C). The

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*Restriction sites are underlined.
ducks were housed in cages with a 12 h light/dark cycle and free access to food and water during this study. To minimize suffering, dying ducks were euthanized humanely with an intravenous injection of sodium pentobarbital at a dose of 120 mg kg$^{-1}$.

**Assessment of virulence in vivo**

Virulence of strains APCE94, APCE94ΔetrA and APCE94CΔetrA was investigated in duck models. Samples of each strain were taken at the exponential growth phase, washed and resuspended in PBS to the appropriate doses. Seven-day-old ducks were randomly divided into four groups and infected intratracheally with $10^8$ colony-forming units (c.f.u.s) of each bacterial strain or PBS. Mortality was monitored daily and, when necessary, ducks were humanely euthanized according to animal welfare guidelines until completion of the experiment at 7 days post-infection.

**Determination of bacterial loads in vivo**

To determine bacterial colonization and proliferative capacity, bacterial loads in the lung, liver and spleen of infected ducks were measured as described previously [21, 27, 28]. Briefly, ducks were infected intratracheally with $10^8$ c.f.u.s of each bacterial strain and euthanized humanely at 24 h post-infection. Lung, liver and spleen were collected, weighed and triturated. Serial dilutions of the homogenates were plated onto LB agar plates and the number of bacteria were counted.

**Quantitative real-time PCR**

The expression of bacterial virulence genes and genes encoding the pro-inflammatory cytokines interleukin (IL)-1β and IL-8 in infected HD-11 cells was investigated by quantitative real-time PCR (qRT-PCR) as described previously [21, 27]. Briefly, bacteria were collected at the logarithmic phase and HD-11 cells were collected at 3 h post-infection. RNA isolation, DNA contamination removal and cDNA synthesis were performed according to the manufacturer's protocol. qRT-PCR was performed to analyse transcription levels using SYBR Premix Ex Taq (TaKaRa, Dalian, China) and gene-specific primers (Table 2). Relative gene expression was normalized to the housekeeping gene dnaE or β-actin. The specific amplification of each gene was confirmed by melt curve analysis. Experiments were performed three times in duplicate.

**Statistical analyses**

Statistical analyses were conducted using the GraphPad Software package (GraphPad Software). One-way analysis of variance (ANOVA) was used to analyse bacterial adherence and invasion data. Two-way ANOVA was used to analyse bacterial survival and qRT-PCR results. Animal infection data were analysed using the non-parametric Mann–Whitney U test. $P<0.05$ was considered statistically significant.

**RESULTS**

**Inactivation of EtrA has no effect on the growth, motility or serum resistance of APCE94**

Previous studies have shown that the epr and epa operons of ETT2 are transcribed and functional [19]. The etra gene is conserved among different ETT2 isoforms, which is located between the epr and epa operons. Amino acid sequence analysis showed that EtrA contains a typical helix-turn-helix (HTH) motif that is 40% identical to the LuxR superfamily regulator GerE (Fig. S1a, available in the online Supplementary Material). The predicted 3D structure of the EtrA HTH motif consists of three helical domains, which make up the major DNA binding motif (Fig. S1b).

The etra mutant and complemented strains were constructed and characterized. To determine whether etra gene deletion had a polar effect on the transcription levels of eprH and epaS, their expression in APCE94ΔetrA and APCE94 strains was analysed by qRT-PCR. No polar effect was shown following the expression of upstream or downstream genes (Fig. S2a). Moreover, no significant difference was observed in the growth rate (Fig. S2b) or the halo diameter (Fig. S2c) among strains APCE94, APCE94ΔetrA and APCE94CΔetrA. Therefore, deletion of etra had no effect on the growth or motility of APCE94.

Bactericidal assays were performed to determine whether etra is involved in bacterial resistance to SPF chicken serum. Strains APCE94, APCE94ΔetrA and APCE94CΔetrA showed similar resistance to SPF chicken serum, indicating that the etra gene is not involved in resistance to bactericidal serum (Fig. 1).

**Deletion of etra reduces intracellular survival of APCE94 in avian HD-11 macrophages**

Bacterial adherence to and invasion of host cells are essential for microbial pathogenesis. Thus, the adherence and invasion capacities of strains APCE94, APCE94ΔetrA and APCE94CΔetrA in avian DF-1 cells were investigated. Although adherence and invasion were slightly increased in the mutant strain APCE94ΔetrA compared to the wild-type strain APCE94, the difference was not statistically significant ($P>0.05$) (Fig. 2a).

The role of EtrA in intracellular survival and replication of APEC was determined. Similar to results of experiments with DF-1 cells, the invasiveness of the mutant strain APCE94ΔetrA in HD-11 cells was higher than that of the wild-type strain APCE94, but the difference was not statistically significant ($P>0.05$). In contrast, at all time points tested, the mutant strain APCE94ΔetrA showed impaired growth and survival in HD-11 cells compared with the wild-type strain APCE94. Moreover, the difference in intracellular survival and replication between APCE94ΔetrA and APCE94 was statistically significant at 24 h p.i. ($P<0.01$). Invasion and intracellular survival were restored in the complemented strain APCE94CΔetrA (Fig. 2b). Taken together,
these findings suggest that EtrA is required for the intracellular survival of APEC in chicken macrophages.

**EtrA is required for bacterial virulence and survival in vivo**

The influence of EtrA on virulence of APEC was evaluated using a duck infection model. Results showed that the mortality rates of ducks infected with strains APCE94, APCE94ΔetrA and APCE94CΔetrA were 87.5% (7/8), 37.5% (3/8) and 62.5% (5/8), respectively (Fig. 3a). These results indicate that EtrA plays a role in APEC virulence.

The bacterial loads of strains APCE94, APCE94ΔetrA and APCE94CΔetrA in infected lung, liver and spleen were assessed. Results showed that deletion of etrA significantly decreased bacterial survival in the liver and spleen (P<0.05), but not in the lung. Moreover, bacterial colonization in the liver and spleen was restored in the complemented strain (Fig. 3b), indicating that EtrA plays a role on the APEC survival during systemic infection.

**Expression profiling of virulence genes**

Previous studies have indicated that regulators within the ETT2 cluster can affect the expression of LEE and ETT2 genes and indirectly regulate the virulence of intestinal pathogenic *E. coli* [16]. Thus, the expression of ETT2 and virulence genes in strains APCE94, APCE94ΔetrA and APCE94CΔetrA was measured by qRT-PCR. Results showed no differences in the expression of ETT2 genes among these three strains (Fig. 4a). However, expression of virulence genes *ompA* and *tsh* was downregulated, and fimbriae genes *fimC* and *mat* were upregulated in the mutant strain APCE94ΔetrA compared to the wild-type strain APCE94 (P<0.01 or P<0.05). The expression of these genes was restored to wild-type levels in the complemented strain APCE94CΔetrA (Fig. 4b).

**Determination of pro-inflammatory cytokine gene expression in HD-11 cells**

To determine whether EtrA affects the host inflammation response, avian HD-11 cells were infected with strains APCE94, APCE94ΔetrA or APCE94CΔetrA, and expression of the genes encoding the pro-inflammatory cytokines IL-1β and IL-8 was assessed by qRT-PCR. The results showed that expression of both genes was significantly upregulated in HD-11 cells infected with the mutant strain APCE94ΔetrA compared to the wild-type strain APCE94 (P<0.05 and P<0.01, respectively), and expression levels were restored to the wild-type levels in HD-11 cells infected with the complemented strain APCE94CΔetrA (Fig. 5). Thus, EtrA suppresses the expression of IL-1β and IL-8 in HD-11 cells infected with APCE94.

**DISCUSSION**

To adapt to new environments, bacteria can rapidly regulate gene expression via multiple regulatory networks [29]. It has previously been shown that ETT2 is widely distributed among *E. coli* strains [8–10, 12, 13, 30]. Although many ETT2 clusters are disrupted or incomplete, it has been shown that ETT2 and its components are involved in bacterial virulence [16, 19–21]. Genomic studies have identified several transcriptional regulators, including Ygeh, YgeK/ EtrB, Etra and EivF, within ETT2 clusters [16, 17]. In
particular, the regulator EtrA is highly similar in all ETT2 cluster isoforms. Amino acid sequence analysis of EtrA proteins has shown that EtrA contains an HTH motif at the C-terminal with 40% identity to the LuxR family regulator GerE, indicating that EtrA can be classified as a member of the LuxR/GerE family of regulators [31]. However, no possible cognate response regulator to EtrA has been identified within the ETT2 cluster. Previous studies have indicated that the regulator EtrA exerts profound negative effects on LEE transcription and T3SS secretion that impact adherence and virulence of EHEC O157:H7 [16]. Although the E. coli pathovars share many virulence strategies,
pathogenicity mechanisms vary between diarrhoeagenic E. coli and ExPEC [32]. Therefore, the function of the ETT2 regulator EtrA in the ExPEC strain requires further examination.

Previous studies have shown that ETT2 regulators can alter the expression of genes within both the ETT2 locus and the LEE locus in EHEC O157:H7 [16]. Such cross-talk between these two E. coli T3SSs prevents unnecessary concurrent expression of multiple T3SS. These regulators include YgeH, a transcriptional activator that is essential for the expression of ETT2 genes but does not regulate other genes [18]. In contrast, regulators EtrA and EivF repress LEE expression and bacterial adhesion capacity [16], whereas the regulator YgeK/EtrB activates LEE expression and promotes A/E lesion formation [17].

Unlike EHEC, ExPEC strains, including APEC, do not contain an LEE locus. Thus, we determined the influence of EtrA on the expression of ETT2 and virulence genes in APEC. Our results indicated that disruption of the \(etrA\) gene did not influence ETT2 expression. However, the transcriptional levels of the virulence genes \(ompA\), \(fimC\), \(mat\) and \(tsh\) were affected by disruption of \(etrA\). The bacterial surface component OmpA plays an important role in environmental adaptation and APEC survival and virulence [33]. In our study, the transcription level of virulence gene \(ompA\) was significantly decreased in the mutant strain APCE94\(\DeltaetrA\). This may explain the defects we observed in bacterial intra-macrophage survival, as well as the proliferation and virulence in a duck model. In contrast, inactivation of \(etrA\) led to significant upregulation of the fimbria genes \(fimC\) and \(mat\), and downregulation of the adhesin gene \(tsh\).
Fimbriae and adhesin are involved in the first step of infection, facilitating the initial interaction between the pathogen and its host's cells [34–36]. Thus, the bacteria may have compensated for the effect of *etrA* deletion on adhesion factors and this could explain why we did not observe a significant difference in adherence or colonization among strains APCE94, APCE94ΔetrA and APCE94CΔetrA in DF-1 cells.

Similarly, another ETT2 regulator, YgeK/EtrB, represses the expression of fimbriae in EAEC strains. Additionally, the regulator YgeK/EtrB plays roles not only through directly interacting with the ler regulatory region but also by repressing *eivF* and *etrA* expression [17]. Moreover, the expression of LEE is co-regulated by EtrA, EivF and YgeK/EtrB. Thus, pathogenic bacteria can effectively modulate gene expression to ensure efficient infection [37].

Bacterial infection triggers an extensive innate immune response in the host. However, some pathogenic bacteria are able to effectively subvert host inflammatory and immune responses through bacterial surface components and effector proteins. Pro-inflammatory cytokines, early signalling molecules in innate immunity, are critical for bacterial clearance [38, 39]. We found that the expression of the genes encoding the pro-inflammatory cytokines IL-1β and IL-8 was significantly increased in cells infected with the mutant strain APCE94ΔetrA compared with the wild-type strain APCE94. This might explain the impaired survival, proliferation and virulence of the mutant strain in ducks. Since fimbriae can induce pro-inflammatory cytokines in host cells after bacterial infection [40, 41], the increased expression of fimbriae that we observed might explain the upregulation of IL-1β and IL-8 genes. Interestingly, similar fimbriae and pro-inflammatory cytokine gene expression patterns have been reported in the ETT2 ATPase gene *eivC* mutant strain [21]. Thus, ETT2 components may alter the bacterial surface structure and expression of virulence genes during infection, leading to improved fitness and virulence of *E. coli*. However, since an ETT2 effector has not yet been identified, it remains unknown if and how these ETT2 genes interfere with the host innate immune response.

**Fig. 4.** Quantification of virulence gene expression. qRT-PCR was performed to measure the expression levels of ETT2-related genes (a) and virulence genes (b) in strains APCE94, APCE94ΔetrA and APCE94CΔetrA. Data were normalized to the housekeeping gene *dnaE*. Results are shown as relative expression ratios compared to expression in the wild-type strain APCE94. Statistical significance was assessed using two-way ANOVA (*, *P*<0.05; **, *P*<0.01).

**Fig. 5.** Cytokine expression in APEC-infected avian HD-11 cells. Expression of IL-1β and IL-8 genes in HD-11 cells infected with strains APCE94, APCE94ΔetrA and APCE94CΔetrA was analysed at 3 h post-invasion by qRT-PCR. Data were normalized to β-actin. Samples were calibrated to gene expression in HD-11 cells infected with wild-type strain APCE94. Expression of genes encoding IL-1β and IL-8 was significantly upregulated in cells infected with strain APCE94ΔetrA compared with the APCE94 strain. Statistical significance was assessed by two-way ANOVA (*, *P*<0.05; **, *P*<0.01).
In conclusion, the ETT2 regulator EtrA contributes to APEC survival, proliferation and virulence in ducks. Since ETT2 is broadly associated with APEC pathogenicity, further investigation of its molecular mechanisms will help to prevent poultry colibacillosis and human infections [42, 43].

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
All animal experiments were conducted in strict accordance with the Guiding Principles for the Humane Treatment of Laboratory Animals, as issued by Ministry of Science and Technology of the People’s Republic of China (Policy No. 2006 398). The experiments described in this study were approved by the Institutional Animal Care and Use Committee at the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences (permit No: Shvri-Po-0244).

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