Identification of ireA, 0007, 0008, and 2235 as TonB-dependent receptors in the avian pathogenic Escherichia coli strain DE205B

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Abstract

Avian pathogenic Escherichia coli (APEC), a pathotype of extraintestinal pathogenic E. coli, causes one of the most serious infectious diseases of poultry and shares some common virulence genes with neonatal meningitis-associated E. coli. TonB-dependent receptors (TBDRs) are ubiquitous outer membrane β-barrel proteins; they play an important role in the recognition of siderophores during iron uptake. Here, in the APEC strain DE205B, we investigated the role of four putative TBDRs—ireA, 0007, 0008, and 2235—in iron uptake. Glutathione-S-transferase pulldown assays indicated that the proteins encoded by these genes directly interact with TonB. Moreover, the expression levels of all four genes were significantly upregulated under iron-depleted conditions compared with iron-rich conditions. The expression levels of several iron uptake-related genes were significantly increased in the ireA, 0007, 0008, and 2235 deletion strains, with the upregulation being the most prominent in the ireA deletion mutant. Furthermore, iron uptake by the ireA deletion strain was significantly increased compared to that by the wild-type strain. Moreover, a tonB mutant strain was constructed to study the effect of tonB deletion on the TBDRs. We found that regardless of the presence of tonB, the expression levels of the genes encoding the four TBDRs were regulated by fur. In conclusion, our findings indicated that ireA, 0007, 0008, and 2235 indeed encode TBDRs, with ireA having the most important role in iron uptake. These results should help future studies explore the mechanisms underlying the TonB-dependent iron uptake pathway.

Introduction

Avian pathogenic Escherichia coli (APEC), a pathotype of extraintestinal pathogenic E. coli (ExPEC), causes serious infectious diseases in poultry [1, 2]. Different serotypes of APEC cause local or systemic infectious diseases in poultry, including respiratory infections, sepsis, polyserositis, coligranuloma, cellulitis, yolk sac infection, omphalitis, and swollen head syndrome, resulting in significant economic losses to the poultry industry [3]. Strains of APEC and neonatal meningitis-associated E. coli (NMEC, a subpathotype of ExPEC), the latter of which causes infections in humans, reportedly share some common virulence genes [4–6]. It is thus particularly important to study the genes encoding virulence factors in APEC strains. These strains contain several virulence-associated genes that encode various virulence factors, including adhesins (fimC, ompA, papC), invasins (ibeA), avian haemolysins (hlyF), serum survival proteins (iss, ompT), and siderophores (iutA, fyuA, iroN); moreover, they show the presence of a pathogenicity island [7–9].

Iron is a vital micronutrient that regulates enzyme activity and metabolism. This element plays a key role in basic cellular processes, such as cellular respiration,
DNA replication, and electron transport and is accordingly essential for bacterial survival in host tissues [10–12]. Furthermore, iron is a necessary growth factor for bacteria and is reportedly involved in the expression of bacterial virulence factors [13]. Iron uptake factors evidently play a pivotal role in *E. coli* growth and pathogenesis. Bacteria employ different strategies to absorb iron from their environment, including siderophore-mediated iron uptake, which occurs on the cell surface [14]. In addition, bacteria either excessively reduce the external pH or dissolve iron oxide to meet their iron requirements by reducing ferric iron to a relatively soluble ferrous form. Another common strategy is to synthesize and secrete iron chelators, such as siderophores, as intracellular iron (Fe²⁺) is rarely found in natural conditions, and Fe²⁺ can be readily oxidized to Fe³⁺ in the presence of oxygen and water [15]. Siderophores then combine with the available iron (Fe³⁺) to form an iron–siderophore complex, which binds to specific receptor proteins on the bacterial cell surface, consequently entering cells via the TonB-dependent transport system, followed by iron release. Siderophore-mediated ferric uptake requires specific outer membrane (OM) receptors, such as FhuA, FecA, and FepA, which reportedly exist in *E. coli* [16]. These OM receptors share the same structural properties and acquire energy by coupling with TonB proteins located in the inner membrane, and they are referred to as TonB-dependent receptors (TBDRs) [17].

TBDRs are known to actively transport ferric–siderophore complexes in Gram-negative bacteria, and they also transport diverse antibiotics, vitamins, nickel complexes, and carbohydrates [18–20]. Transporters involved in iron uptake have very strict siderophore selectivity. A strong correlation exists between the amount of iron and siderophores that bacteria can use and the number of genes encoding iron-regulated TBDRs [19]. In simple terms, iron depletion triggers the upregulation of genes encoding TBDRs.

In this study, upon analysing the whole genome of the APEC strain DE205B, we identified six putative TBDRs; however, the construction of two mutants failed. Thus, we eventually investigated the roles of four putative TBDRs—**ireA**, **0007**, **0008**, and **2235**—in iron uptake.

### Materials and methods

**Bacterial strains, plasmids, and growth conditions**

The bacterial strains and plasmids used in this study are listed in Table 1. The APEC strain DE205B (O2:K1), which was isolated from a duck with neurological symptoms, was previously determined to have no antibiotic (ampicillin, kanamycin, and chloramphenicol) resistance [21–25]. All the APEC strains used in this study were cultured in Luria–Bertani (LB, Oxoid, Thermo-Fisher,

| Strain or plasmid | Characteristics | References |
|-------------------|----------------|------------|
| Strain            |                |            |
| DE205B            | O2:K1, NalR    | [21–25]    |
| DE205BΔ*ireA      | ireA Deletion mutant strain | [21]        |
| DE205BΔ*ireA/*ireA* | ireA Complemented strain | [21]        |
| DE205BΔ0007       | 0007 Deletion mutant strain | This study |
| DE205BΔ0007/0007* | 0007 Complemented strain | This study |
| DE205BΔ0008       | 0008 Deletion mutant strain | This study |
| DE205BΔ0008/0008* | 0008 Complemented strain | This study |
| DE205BΔ2235       | 2235 Deletion mutant strain | This study |
| DE205BΔ2235/2235* | 2235 Complemented strain | This study |
| DE205BΔtonB       | tonB deletion mutant strain | This study |
| DE205BΔtonB/tonB* | tonB Complemented strain | This study |
| DH5a              | Competent invitrogen cells | Vazyme     |
| BL21              | Competent invitrogen cells | Vazyme     |
| Plasmid           |                |            |
| pKD46             | Amp, expresses λ red recombinase | [26]        |
| pKD4              | Kan, template plasmid | [26]        |
| pSTV28            | Cm, expression using lac promotor | TakaRa     |
| pCP20             | Cm, Amp, yeast Flp recombinase gene, FLp | [26]        |
| pET32a            | Amp, expresses a fusion fragment of the His tag | This study |
| pGEX-4t-1         | Amp, expresses a fusion fragment of the GST tag | This study |
USA) or solid LB medium supplemented with 2% agar, followed by incubation in a shaker at 37 °C and 180 rpm unless otherwise stated. For culture of the mutant strain, appropriate antibiotics (ampicillin, 100 μg/mL; kanamycin, 50 μg/mL; chloramphenicol, 15 μg/mL) were added to the LB media. M9 medium was prepared as follows: 200 mL of 5 x M9 salt solution (12.8 g Na2PO4·7H2O, 3.0 g KH2PO4, 0.5 g NaCl, and 1.0 g NH4Cl) was mixed with 2 mL of 1 M MgSO4, 20 mL of 20% glucose, and 0.1 mL of 1 M CaCl2. This solution was then dissolved in 1000 mL double-distilled water and filtered through a 0.22-μm membrane. Fe (0.1 mM FeCl3·6H2O) was added or not added to M9 medium to establish iron-rich or iron-depleted conditions, respectively.

Construction of the mutant and complemented strains

For analysis of the role of the four TBDRs in iron uptake, three genes encoding TBDRs (0007, 0008, 2235) were knocked out, and the ireA mutant strain, which was previously constructed [21], was used in this study. A single mutant strain for each of the three genes (0007, 0008, 2235) was constructed using the Red homologous recombination method in E. coli [26]. Briefly, for generation of the 0007 mutant strain, a gene-targeting fragment containing a homologous arm on both sides of 0007 was amplified by PCR using the plasmid pKD4 as the template, which contains the phage λ Red system under control of an arabinose promoter. Competent DE205B cells containing pKD46 were then prepared; L-arabinose was added to induce the expression of the phage λ Red system, and the target fragments were transformed by electroporation into DE205B to replace 0007 with the resistance gene. The recombinant construct was screened by growth on LB plates supplemented with kanamycin and identified by cross-PCR. Details of the primers (0007-F/0007-R, K1, K2) used for amplification are listed in Additional file 1. Next, the temperature-sensitive plasmid pCP20 was transformed into the recombinant strain to remove the resistance gene. Finally, pCP20 was removed by growth at 42 °C for 24 h to obtain the mutant strain DE205BΔ0007 that did not show any resistance. For analysis of the effect of tonB deletion on the TBDRs, a tonB mutant strain was also constructed using the same method. Similarly, the 0008, 2235, and tonB mutant strains, namely, DE205BΔ0008, DE205BΔ2235, and DE205BΔtonB, respectively, were obtained.

The construction of the complemented strain involved recovering the deleted gene. The genomic DNA of DE205B was used as the template to amplify 0007, including its putative promoter gene. The PCR product was then purified and inserted into pSTV28, and the composite vector PSTV28-0007 was transformed by electroporation into DE205BΔ0007 to produce the complemented strain DE205BΔ0007/0007. In the same manner, the complemented strains DE205BΔ0008/0008*, DE205BΔ2235/2235*, and DE205BΔtonB/tonB* were obtained. The ireA deletion strain DE205BΔireA and the complemented strain DE205BΔireA/ireA* were constructed in an earlier study and used in this study as well [21].

Growth curves

Growth curves of the wild-type (WT), mutant (DE205BΔireA, DE205BΔ0007, DE205BΔ0008, DE205BΔ2235) and complemented strains (DE205BΔireA/ireA*, DE205BΔ0007/0007*, DE205BΔ0008/0008*, DE205BΔ2235/2235*) cultured in LB, iron-depleted M9 and iron-rich M9 media were constructed. All strains were grown overnight in 5 mL of LB medium supplemented with antibiotics, as appropriate. The cultured strains were centrifuged at 5000 rpm for 10 min to remove the supernatant, and the cell pellets were washed twice with PBS containing 200 mM 2,2’-dipridyl; the optical density at 600 nm (OD600) was adjusted to 1. Then, 100 μL of the suspension was inoculated into 50 mL of LB medium, and 1000 μL of this solution was inoculated into 50 mL of iron-depleted or iron-rich M9 medium. The OD600 of the LB culture was measured using a spectrophotometer (Philes, Nanjing, China) every hour for a total of 10 h and that of the iron-depleted and iron-rich M9 cultures was measured at 0, 4, 8, 12, 24, and 48 h [27].

Gene expression analyses

As ireA, 0007 (GI: MN239889), 0008 (GI: MN239890), and 2235 (GI: MN239892) encode putative TBDRs, their expression levels were determined under iron-rich and iron-depleted conditions. DE205B was cultured to the mid-log phase in both types of M9 media, and the gene expression levels were determined using quantitative real-time PCR (qRT-PCR). Briefly, total RNA was extracted from bacteria grown under different culture conditions using a bacterial RNA kit (Omega Bio-Tek, Beijing, China) and reverse transcribed into cDNA using the PrimeScript™ RT reagent Kit (TaKaRa, Perfect Real Time, Japan) according to the manufacturer’s instructions. qRT-PCR was performed in a 20 μL reaction volume containing SYBR Green PCR Master Mix (Vazyme Biotech, Nanjing, China) and 0.1 μM primers (Additional file 1) specific to the iron uptake-related genes (0007, 0008, 2235, ireA, fecA, fluA, iutA, iroN1, iroN2); the quantification data were analysed with ABI StepOne Software, version 2.3 (USA). For analysis of the effect of gene deletion on the ferric uptake system, the expression levels of iron uptake-related genes (fecA, fluA, fecA, fecB, iutA, chuA, chuA, chuA, chuA, chuA, chuA) were tested in LB medium. Total RNA was extracted from the WT, mutant (DE205BΔireA, DE205BΔ0007, DE205BΔ0008, DE205BΔ2235), and complemented (DE205BΔireA/ireA*, DE205BΔ0007/0007*,
Expression of the TBDRs

The pertinent gene fragments (ireA, 0007, 0008, 2235) were inserted into the plasmid pET32a, and tonB was cloned into pGEX-4t-1. Proteins were expressed in BL21 (Vazyme Biotech) via addition of 1 mM isopropyl β-D-1-thiogalactopyranoside in the mid-log phase. The recombinant proteins IreA, 0007, 0008, and 2235 expressed in vitro carried a His tag, and TonB carried a glutathione-S-transferase (GST) tag. The primer details are listed in Additional file 1. The His-tag fusion proteins were purified by Ni-column chromatography, and the biological activity of the proteins in inclusion bodies was restored via a conventional method that involves using a urea-containing protein refolding solution (including 20 mM Tris–HCl, 1 mM GSH, 0.2 mM GSSG, 0.5 M NaCl). Finally, we concentrated and collected the putative TBDRs (IreA, 0007, 0008, 2235) with His tags by centrifugation in an ultracentrifugal tube (10 kDa) at 4 °C. The final fusion proteins were thus obtained (34 kDa for 0007-His, 66 kDa for 0008-His, 95 kDa for 2235-His, and 93 kDa for IreA-His).

GST pulldown assay

The functional domain of TonB (residues 150–239) is critical, as it directly interacts with the OM receptor [31]. As such, to further verify whether the four putative TBDRs directly interact with TonB, we performed GST pulldown assays. Cells expressing the GST fusion proteins were lysed using an ultrasonic cell disrupter system (Thermo Fisher Scientific, USA). Unlysed cells and impurities were removed by centrifugation (5000 rpm, 10 min), and the supernatant was incubated with GST-binding beads (Enriching Biotechnology, Ltd., Shanghai, China) for 3 h, followed by stringent washing with PBS (2 mM KH₂PO₄, 2.6 mM KCl, 8 mM Na₂HPO₄, 136 mM NaCl) to minimize nonspecific binding. Next, the samples were incubated with the putative TBDRs (IreA, 0007, 0008, 2235) with His tags. Samples were collected after multiple washes with PBS to minimize nonspecific binding. Finally, protein separation was performed by SDS-PAGE on 12% protein gels (Warbio, Shanghai, China), followed by protein detection via a Western blot assay with His-tagged monoclonal antibodies [32, 33]. Further, pGEX-4t-1 (no gene fragment inserted) was incubated with the putative TBDRs, and this was used as the negative control to exclude nonspecific binding of prey proteins to the beads or to GST itself.

Iron uptake test

To verify whether the four TBDRs are directly involved in iron uptake, we determined the Fe concentrations in the WT, mutant, and complemented strains of DE205B using an iron colorimetric assay kit (Elabscience Biotech, Wuhan, China, catalogue no. E-BC-K139-S). The WT, mutant (DE205BΔireA, DE205BΔtonB, DE205BΔ0007, DE205BΔ0008, DE205BΔ2235), and complemented (DE205BΔireA/ireA*, DE205BΔtonB/tonB*, DE205BΔ0007/0007*, DE205BΔ0008/0008*, DE205BΔ2235/2235*) strains were incubated overnight in 5 mL of LB medium supplemented with antibiotics, as appropriate. The cultured strains were then centrifuged (5000 rpm, 10 min), and the supernatant was discarded. The obtained cell pellets were washed twice with PBS containing 200 M 2,2′-dipyridyl, and the OD₆₀₀ was adjusted to 1. Then, 1000 μL of the suspension was inoculated into 100 mL of LB media. Briefly, the strains were grown to the log phase and washed three times with 0.9% NaCl. Cell pellets were subsequently obtained and suspended in PBS, and the cells were lysed using an ultrasonic cell disrupter system (Thermo Fisher Scientific, USA) to release intracellular iron. Finally, impurities were removed by centrifugation (5000 rpm, 10 min); a sample of the supernatant was used for iron content determination [34]. Deionized water (0.5 mL), iron standard stock solution (0.5 mL), and sample (0.5 mL) were individually mixed with a chromogenic agent (1.5 mL), boiled for 5 min, and then centrifuged at 2300 rpm for 10 min; the supernatant was subsequently collected. According to the manufacturer’s instructions for the iron colorimetric assay kit, iron content was estimated by measuring the OD at 520 nm (OD₅₂₀) of the supernatant. The following formula was used:

\[
\text{Fe concentration (μg/L) = } \left( \frac{\text{sample OD}_{520} - \text{deionized water OD}_{520}}{\text{iron standard stock solution OD}_{520}} \right) \times \text{standard iron concentration (2000μg/L)}
\]
Statistical analyses
Statistical analyses were performed in GraphPad Prism 7.0 using unpaired t-tests [35]. In the figures, the error bars indicate the standard deviations, “*” represents $P<0.05$, “**” represents $P<0.01$, and “***” represents $P<0.001$. qRT-PCR data from three individual experiments were used to determine the differences (fold change) in gene transcription levels. Similarly, in the growth curve and iron uptake analyses, each reaction was performed three times to overcome any experimental errors.

Results
Iron depletion upregulated the expression levels of the genes encoding the putative TBDRs
The expression levels of all four genes were significantly upregulated under iron-depleted conditions compared with iron-rich conditions; the expression levels of 0007, 0008 and 2235 were upregulated 1.76 times ($P<0.001$), 1.43 times ($P<0.05$), and 1.71 times ($P<0.01$), respectively. The ireA gene was the most upregulated (1.87 times, $P<0.001$) (Figure 1). In addition, the expression levels of the confirmed iron-uptake genes were upregulated under iron-depleted conditions compared to iron-rich conditions: the expression levels of fecA and fluA were significantly upregulated ($P<0.01$), but those of iutA, iroN1, and iroN2 were not significantly changed (Figure 1).

Iron depletion decreased the growth rate of the mutant strains
Growth curves of the WT and mutant strains cultured in LB, iron-depleted M9 and iron-rich M9 media were constructed. The obtained results indicated that the growth rates of the mutant strains (DE205BΔireA, DE205BΔ0007, DE205BΔ0008 and DE205BΔ2235) were comparable to that of the WT strain in LB medium (Figure 2A). Under iron-depleted conditions, the growth rates of the mutant strains were slightly lower than that of the WT strain (Figure 2B). However, under iron-rich conditions, the growth rates of the mutant strains were comparable to that of the WT strain (Figure 2C).

Compensatory expression of other iron uptake-related genes was significantly upregulated in the mutant strains
Most iron uptake-related genes in DE205BΔireA, including fecA, fhuA, fepA, fepC, feoB, and fyuA, were significantly upregulated ($P<0.05$); the expression levels of all these genes were restored to WT levels in the complemented strain DE205BΔireA/ireA* (Figure 3A). In contrast, relative to those of the WT strain, the expression levels of fecA, fhuA, and iroN2 were higher only in DE205BΔ0007, DE205BΔ0008, and DE205BΔ2235, respectively (Figures 3B–D).

IreA, 0007, 0008, and 2235 are TBDRs
Protein–protein interactions between the four putative TBDRs and TonB were analysed in vitro. The SDS-PAGE results showed that these proteins specifically bound to TonB; further, the Western blot assay results revealed the presence of a His tag on the TBDRs. These findings confirmed that TonB positively interacted with all four putative TBDRs (Figures 4A, B).

Iron uptake by DE205BΔireA increased and that by DE205BΔtonB decreased
We found that at the mid-log phase of growth, Fe uptake by DE205BΔireA (Figure 5A) increased ($P<0.05$) compared to that by the WT strain, whereas Fe uptake by DE205BΔtonB (Figure 5B) decreased ($P<0.05$). Fe uptake by the complemented strains was restored to normal. There were no significant differences in Fe uptake between the 0007, 0008, and 2235 mutant strains and the WT strain (Figures 5D–F). In addition, compared to the medium for the WT and complemented strains, the medium in which DE205BΔtonB was cultured turned red (Figure 5C).

The TBDRs are regulated by fur
The expression levels of the genes encoding the TBDRs were examined by qRT-PCR in the WT strain, DE205BΔtonB, and DE205BΔtonB/tonB* under iron-depleted conditions. The ireA expression level in DE205BΔtonB was significantly upregulated ($P<0.01$); furthermore, the expression levels of 0007, 0008, and 2235 were slightly upregulated (Figure 6). In addition, we observed that the expression level of the well-known TBDR fepA was significantly increased.
(P < 0.01), consistent with the results for ireA. Given that the TBDRs are under the control of the ferric uptake regulator fur, the expression levels of fur in the WT strain and DE205BΔtonB under iron-depleted conditions were examined. qRT-PCR showed that relative to that of the WT strain, the expression level of fur in DE205BΔtonB was downregulated (P < 0.01), indicating that the expression of the genes encoding the TBDRs was regulated by fur in the mutant strain DE205BΔtonB (Figure 7).

The expression levels of the genes encoding the four TBDRs in DE205BΔtonB were further tested under iron-rich or iron-depleted conditions using qRT-PCR. The obtained results indicated that the expression level of ireA was upregulated 6.5 times (P < 0.05) under iron-depleted conditions compared to iron-rich conditions and that the expression levels of 0007, 0008, and 2235 were also slightly upregulated (Figure 8). The expression levels of the genes encoding known TBDRs, including fepA, iutA, and feoB, were also significantly increased (P < 0.05).

Discussion

Here, six putative TBDRs were predicted, but deletion or mutant strains could be obtained for only four genes encoding TBDRs (ireA, 0007, 0008, 2235). Motif analyses of ireA sequences have helped reveal the C-terminal region of the TBDRs [12, 13], but experimental verification is lacking. Herein, IreA and TonB were expressed in vitro, and their interaction was determined via GST pulldown assays, which confirmed that ireA is a TBDR. In addition to ireA, three genes (0007, 0008, and 2235) were confirmed to encode TBDRs; however, compared to the expression levels of 0007, 0008, and 2235, that of ireA was the most upregulated in response to iron depletion. Moreover, in the ireA mutant strain DE205BΔireA, iron uptake was increased, consistent with the excessive compensatory expression of other iron uptake-related genes. These findings are similar to those reported for the known TBDR fepA, indicating that ireA might function similarly to fepA [17].

TBDRs transport ferric siderophores on the OM with a lack of energy, relying on TonB to transduce energy
from the proton motive force of the ExbB–ExbD complex in the inner membrane [36, 37]. In this study, a tonB mutant strain was constructed to analyse the effect of tonB deletion on TBDRs. The obtained results indicated that iron uptake by this mutant strain was significantly decreased; the medium used for culturing DE205BΔtonB turned red as the free iron in the culture medium was oxidized. In addition, the expression level of ireA in

![Figure 3](image1.png)

**Figure 3** The expression levels of the iron uptake-related genes. The expression levels of the iron uptake-related genes (fecA, fhuA, fepA, fepC, feoB, fyuA, chuA, iroN1, iroN2) were tested by qPCR. A. The expression levels of the iron uptake-related genes in the WT strain, DE205BΔireA, and DE205BΔireA/ireA*. B. The expression levels of the iron uptake-related genes in the WT strain, DE205BΔ0007, and DE205BΔ0007/0007*. C. The expression levels of the iron uptake-related genes in the WT strain, DE205BΔ0008, and DE205BΔ0008/0008*. D. The expression levels of the iron uptake-related genes in the WT strain, DE205BΔ2235, and DE205BΔ2235/2235*. The relative gene expression levels were calculated using the 2−ΔΔCt method; the values are expressed as percentages.

![Figure 4](image2.png)

**Figure 4** GST pulldown assay. In vitro GST pulldown assays indicating interactions between TonB and the putative TBDRs (ireA, 0007, 0008, 2235) were performed. Incubation of pGEX-4t-1 (no gene fragment inserted) with a putative protein was used as a negative control. A. SDS-PAGE. TBDRs with the His tag (TBDRs-His) are highlighted using black boxes; B. Western blot. All His tags are marked with black boxes. M: protein marker (kDa); 1: TonB+0007; 2: pGEX-4t-1+0007; 3: TonB+0008; 4: pGEX-4t-1+0008; 5: TonB+2235; 6: pGEX-4t-1+2235; 7: TonB+ireA; and 8: pGEX-4t-1+ireA.
DE205BΔtonB was significantly upregulated and that of fur was downregulated. Due to severe iron uptake defects, an iron-depleted environment was created, causing fur to upregulate the expression of the TBDRs in DE205BΔtonB, which implies that the TBDRs are regulated by fur and not directly affected by the presence or absence of tonB.

ireA was initially found in ExPEC, predicted to be a TBDR, and believed to play a role in the colonization of E. coli by functioning as a receptor on the OM [13]. In this study, ireA was indeed shown to be a TBDR. Our previous research has proven that ireA also plays a role in adhesion and stress resistance in APEC [13, 21]. Adhesion is the first step in APEC colonization, and the development of a receptor antagonist could protect against the establishment of a bacterial infection.
As a multifunctional receptor, IreA may have potential for vaccine development. Each TBDR is composed of a 22-strand β-barrel and an N-terminal TonB box domain with which TonB interacts [38–40]. TonB interacts in vivo with the TonB box of TBDRs (FepA, FhuA, FecA) to form disulphide bonds and eventually transport iron into cells. TBDRs specifically recognize iron carriers. However, the TonB domain of IreA has not yet been identified, and the iron siderophore structure to which it binds has not been studied either. Future research should focus on investigating these issues.

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Authors’ contributions
FT and JD designed the experiments. ZZ wrote the manuscript and performed most of the experiments described in the manuscript. SJ, YL, YS, PY, QG, HZ, and YL provided help during the experiments. FX, XZ, and JR provided valuable suggestions on the manuscript. All authors read and approved the final manuscript.

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Supplementary information
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Additional file 1: Primers used in this study.

Abbreviations
APEC: avian pathogenic Escherichia coli; OM: outer membrane; LB: Luria–Bertani medium; OD600: optical density at 600 nm; qRT-PCR: quantitative real-time polymerase chain reaction; GSH: glutathione, r-glutamyl cysteine + glycine; GSSH: oxidized glutathione; GST: glutathione-S-transferase; WT: wild-type.

Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
The datasets analysed during the current study are available upon request from the corresponding author.

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References
1. Kaper JB, Nataro JP, Mobley HL. (2004) Pathogenic Escherichia coli. Nat Rev Microbiol 2:123–140
2. Croxen MA, Finlay BB (2010) Molecular mechanisms of Escherichia coli pathogenicity. Nat Rev Microbiol 8:26–38
3. Markland SM, LeStrange KJ, Sharma M, Knel KE (2015) Old friends in new places: exploring the role of extraintestinal E. coli in intestinal disease and foodborne illness. Zoonoses Public Health 62:491–496
4. Rodriguez-Siek KE, Giddings CW, Doetkott C, Johnson TJ, Fakhr MK, Nolan LL (2005) Comparison of Escherichia coli isolates implicated in human urinary tract infection and avian colibacillosis. Microbiology 151:2007–2110
5. Manges AR (2016) Escherichia coli and urinary tract infections: the role of poultry-meat. Clin Microbiol Infect 22:122–129
6. Tivendale KA, Logue CM, Kariyawasam S, Jordan D, Hussein A, Li G, Wannemuehler Y, Nolan LL (2010) Avian-pathogenic Escherichia coli strains are similar to neonatal meningitids E. coli strains and are able to cause meningitis in the rat model of human disease. Infect Immun 78:3412–3419
7. Ewers C, Antao EM, Diehl I, Philipp HC, Wieler LH (2009) Intestine and environment of the chicken as reservoirs for extraintestinal pathogenic Escherichia coli strains with zoonotic potential. Appl Environ Microbiol 75:184–192
8. Li G, Latumus C, Ewers C, Wieler LH (2005) Identification of genes required for avian Escherichia coli septicemia by signature-tagged mutagenesis. Infect Immun 73:2818–2827
9. Dho-Moulin M, Fairbrother JM (1999) Avian pathogenic Escherichia coli (APEC). Vet Res 30:299–316
10. Andrews SC, Robinson AK, Rodriguez-Quinones F (2003) Bacterial iron homeostasis. FEMS Microbiol Rev 27:215–237
11. Huynh C, Andrews NW (2008) Iron acquisition within host cells and the pathogenicity of Leishmania. Cell Microbiol 10:293–300
12. Garenaux A, Houle S, Folch B, Dalldale G, Truesdell M, Lepine F, Doucet N, Dozois CM (2013) Avian lipocalin expression in chickens following Escherichia coli infection and inhibition of avian pathogenic Escherichia coli growth by Ex-FABP. Vet Immunol Immunopathol 152:156–167
13. Russo TA, Carlino UB, Johnson JR (2001) Identification of a new iron-regulated virulence gene, ireA, in an extraintestinal pathogenic isolate of Escherichia coli. Infect Immun 69:6209–6216
14. Khan A, Singh P, Srivastava A (2018) Synthesis, nature and utility of universal iron chelator-siderophore: a review. Microbiol Res 212–213:103–111
15. Kim S, Lee JH, Seok JH, Park YH, Jung SW, Cho AE, Lee C, Chung MS, Kim KH (2016) Structural basis of novel iron-uptake route and reaction intermediates in ferritins from gram-negative bacteria. J Mol Biol 428:5007–5018
16. Koster W (2001) ABC transporter-mediated uptake of iron, siderophores, heme and vitamin B12. Res Microbiol 152:291–307
17. Grescock MG, Postle K (2017) Going outside the TonB box: identification of novel FepA-TonB8 interactions in vivo. J Bacteriol 199:e00649-16
18. Cascales E, Buchanan SK, Duche D, Kleanthous C, Lloubes R, Postle K, Riley M, Slatin S, Cavard D (2007) Colicin biology. Microbiol Mol Biol Rev 71:158–229
19. Schalk J, Mislin GL, Brillet K (2012) Structure, function and binding selectivity and stereoselectivity of siderophore-iron outer membrane transporters. Curr Top Membr 69:37–66
20. Gomez-Santos N, Glatter T, Koebnik R, Swiatek-Polatynska MA, Sogaard-Andersen L (2019) A TonB-dependent transporter is required for secretion.
of protease PopC across the bacterial outer membrane. Nat Commun 10:1360.

21. Li Y, Dai J, Zhuge X, Wang H, Hu L, Ren J, Chen L, Li D, Tang F (2016) Iron-regulated gene ireA in avian pathogenic Escherichia coli participates in adhesion and stress-resistance. BMC Vet Res 12:167.

22. Wang S, Niu C, Shi Z, Xia Y, Yaqoob M, Dai J, Lu C (2011) Effects of ibeA deletion on virulence and biofilm formation of avian pathogenic Escherichia coli. Infect Immun 79:279–287.

23. Wang S, Xia Y, Dai J, Shi Z, Kou Y, Li H, Bao Y, Lu C (2011) Novel roles for autotransporter adhesin AatA of avian pathogenic Escherichia coli colonization during infection and cell aggregation. FEMS Immunol Med Microbiol 63:328–338.

24. Wang S, Shi Z, Xia Y, Li H, Kou Y, Bao Y, Dai J, Lu C (2012) IbeB is involved in the invasion and pathogenicity of avian pathogenic Escherichia coli. Vet Microbiol 159:411–419.

25. Zhuge X, Wang S, Fan H, Pan Z, Ren J, Yi L, Meng Q, Yang X, Lu C, Dai J (2013) Characterization and functional analysis of AatB, a novel autotransporter adhesin and virulence factor of avian pathogenic Escherichia coli. Infect Immun 81:2437–2447.

26. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A 97:6640–6645.

27. Holden KM, Browning GF, Noormohammadi AH, Markham PF, Marenda MS (2012) TonB is essential for virulence in avian pathogenic Escherichia coli. Comp Immunol Microbiol Infect Dis 35:129–138.

28. Ali-Benali MA, Alary R, Joudrier P, Gautier MF (2005) Comparative expression of five Lea Genes during wheat seed development and in response to abiotic stresses by real-time quantitative RT-PCR. Biochim Biophys Acta 1730:56–65.

29. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2−∆∆Ct method. Methods 25:402–408.

30. Wasilewska I, Gupta RK, Palchevska O, Kuznicki J (2019) Identification of zebrafish calcium toolkit genes and their expression in the brain. Genes 10:230.

31. James KJ, Hancock MA, Gagnon JN, Coulton JW (2009) TonB interacts with BtuF, the Escherichia coli periplasmic binding protein for cyanocobalamin. Biochemistry 48:9212–9220.

32. Lu L, Tai G, Hong W (2005) Interaction of Arl1-GTPase with the GRIP domain of golgin-245 as assessed by GST (glutathione-S-transferase) pull-down experiments. Methods Enzymol 404:432–441.

33. Kanno E, Ishibashi K, Kobayashi H, Matsui T, Ohbayashi N, Fukuda M (2010) Comprehensive screening for novel rab-binding proteins by GST pull-down assay using 60 different mammalian Rab5s. Traffic 11:491–507.

34. Ajiboye TO, Skiebe E, Wilharm G (2018) Contributions of ferric uptake regulator Fur to the sensitivity and oxidative response of Acinetobacter baumannii to antibiotics. Microb Pathog 119:35–41.

35. Ling D, Salvaterra PM (2011) Robust RT-qPCR data normalization, validation and selection of internal reference genes during post-experimental data analysis. PLoS One 6:e17762.

36. Devanathan S, Postle K (2007) Studies on colicin B translocation: fepA is gated by TonB. Mol Microbiol 65:441–453.

37. Krewulak KD, Vogel HJ (2011) TonB or not TonB: is that the question? Biochem Cell Biol 89:87–97.

38. Sarver JL, Zhang M, Liu L, Nyenhuis D, Cañoso DS (2018) A dynamic protein-protein coupling between the TonB-dependent transporter FhuA and TonB. Biochemistry 57:1045–1053.

39. Wang Y, Chen X, Hu Y, Zhu G, White AP, Köster W (2018) Evolution and sequence diversity of FhuA in Salmonella and Escherichia. Infect Immun 86:e00573-18.

40. Chimento DP, Kadner RJ, Wiener MC (2005) Comparative structural analysis of TonB-dependent outer membrane transporters: implications for the transport cycle. Proteins 59:240–251.

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