Quorum sensing-1 signaling of \(N\)-hexanoyl-\(\lambda\)-homoserine lactone contributes to virulence in avian pathogenic \textit{Escherichia coli}

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Abstract

Avian pathogenic \textit{E. coli} (APEC) caused avian colibacillosis is mostly common in poultry industry worldwide. APEC virulence factors lead to pathogenesis and the quorum sensing (QS) system is actively involved in the regulation of these virulence factors. Signaling molecules in QS are known as autoinducers (AIs). In QS-1, \textit{E. coli} encodes a single LuxR homolog, i.e., \textit{SdiA}, but does not express the LuxI homolog, an acyl-homoserine lactone (AHL) synthase of producing AI-1. Avian pathogenic \textit{E. coli} (APEC) regulates its virulence genes expression in response to exogenous AHLs, but regulatory mechanisms of AHL and QS-1 are still unknown. This study targeted the APEC CE129 isolate as the reference strain, and the \textit{Yersinia enterocolitica yenI} gene was expressed into APEC CE129. CE129/pyenI was conferred the ability to produce AHL signal. The CE129 \textit{SdiA} mutant strain with an in-frame \textit{sdiA} (AHL receptor) gene deletion was constructed by a \(\lambda\)Red recombination system, which lost the ability to sense AHL. The goal of this study was to explore the function of QS-1 upon virulence and elucidate the regulatory effect of QS-1/AHL signals in the APEC strain. Adherence and invasion assays revealed that QS-1 affected APEC adherence and survival ability. APEC biofilm formation was also suppressed under C6HSL. Interestingly, APEC exhibited different phenotypes of acid tolerance and flagella expression when compared to enterotoxigenic \textit{E. coli} or enterohemorrhagic \textit{E. coli} (ETEC and EHEC, respectively). These findings enhance our understanding of the QS mechanism.

Keywords  APEC · AHL · Quorum sensing · Virulence

Introduction

Avian pathogenic \textit{Escherichia coli} (APEC) is one of the most common pathogens in chickens, turkeys, and other birds (Gao et al. 2016; Wu et al. 2017). It causes severe extra-intestinal infection and leads to avian colibacillosis, the most common bacterial infections of poultry worldwide, which has resulted in great economic losses (Gao et al. 2019). APEC utilizes multiple virulence factors for its colonization and pathogenicity, including fimbrial adhesins, flagella, invasion protein, and iron acquisition systems (Gao et al. 2016).

Quorum sensing (QS) has been reported to play a key role in the regulation of virulence-related genes in APEC (Han et al. 2013; Palaniyandi et al. 2013; Yang et al. 2013; Yang et al. 2014; Cui et al. 2016; Yu et al. 2018). QS is a kind of bacterial communication system that controls the expression of multiple genes in response to bacterial population density. Small chemical signal molecules called autoinducers (AIs) are produced, released, and detected in the QS...
Many bacteria utilize QS to regulate gene expression in response to cell population density to control diverse biological processes, including symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporation, and biofilm formation (Reading and Sperandio 2006; Walters and Sperandio 2006; Boyen et al. 2009; Yang et al. 2013; Yang et al. 2014). Gram-positive and gram-negative bacteria share the QS-II system, which regulates the expression of multiple genes through AI-2. In many pathogenic bacteria, QS-II plays an important role in regulating bacterial virulence strategies, including type III secretion systems, pathogenicity, and biofilm formation (Li et al. 2007; Yang et al. 2014, 2018a, b). The QS-II mutant APEC strain exhibited decreased invasion, reduced lethality in a chicken embryo lethality assay, and diminished survival ability in chicken macrophage cell line derived cells (Han et al. 2013; Palaniyandi et al. 2013). The QS-II mutant APEC DE17 strain displayed a 31.5-fold reduction of virulence in ducklings, with significantly lower mRNA levels of several virulence genes, including iucD, fyuA, vat, ompA, iss, fimC, and tsh (Han et al. 2013). Fu explored the correlation between QS-II and APEC virulence in chicken type II pneumocytes and found that QS-II activity was involved in the cell damage process induced by APEC O78.

Although previous studies detected a correlation between APEC and QS-II, to our knowledge, there are no reports on QS-I function upon APEC virulence. E. coli encodes a single LuxR homolog, SdiA, but does not express the LuxI homolog, acyl-homoserine lactone (AHL) synthase, which produces AI-1. In the presence of AHLs, a considerable proportion of SdiA is expressed in a folded, soluble form in E. coli, as well as in nonfunctional, insoluble inclusion bodies in the absence of AI-1 (Smith et al. 2008; Smith et al. 2011; Dyszel et al. 2010; Yang et al. 2013). Thus, E. coli regulates its virulence gene expression in response to the population density of other AHL-positive bacteria, which is known as “quorum eavesdropping” (Smith et al. 2011). These responses include cell division, antibiotic resistance, motility, and the activity of other QS systems. To further explore the effect of quorum eavesdropping on the virulence of APEC strains CE129 was selected as the reference APEC strain in this study. The recombinant CE129 strain containing the yenl gene from Yersinia enterocolitica was subsequently constructed, which was employed to analyze the effect of endogenous synthetic AHL. yenl synthesizes AHL signals like C6HSL (Atkinson et al. 2006; Yang et al. 2013), which E. coli is sensitive to; thus, C6HSL was used as the reference AHL signal in this study. Commercial C6HSL was exogenously added to the culture medium for CE129 in the target experiment, and the sdiA-deletion mutant strain was constructed for exploring exogenous AHL function upon APEC virulence.

### Materials and methods

#### Bacterial strains

The strains and plasmids used in this study are listed in Table 1. LB broth or LB agar plates were used for bacterial growth. CE129, a clinically isolated APEC strain that has been stored in our laboratory (Ding et al. 2018), was selected

| Table 1 | Strains and plasmids used in this study |
|---------|-----------------------------------------|
| **Strain or plasmid** | **Description** | **Source or reference** |
| CE129 | Non-enteropathogenic E. coli | Stored in this lab |
| CE2 | O78 serotype APEC | Stored in this lab |
| CE11 | O1 serotype APEC | Stored in this lab |
| CE12 | O2 serotype APEC | Stored in this lab |
| CE129/pyenI | CE129 carrying pyenI, Amp<sup>+</sup> | This study |
| CE129/pBR | CE129 carrying pBR322, Amp<sup>+</sup> | This study |
| CE129<sup>ΔsdiA</sup> | sdiA deficient mutant | This study |
| CE129<sup>ΔsdiA/sdiA</sup> | sdiA complement strain | This study |
| ETEC 107/86 | 107/86 carrying pyenl, Amp<sup>+</sup> | (Havens et al. 1992) |
| EHEC 8624 | Wild-type EHEC O157:H7 | Stored in this lab |
| E. coli pSB401 | AHL biosensor | Stored in this lab |
| **Plasmid** | | |
| pBR322 | Expression vector, Amp<sup>+</sup> | Takara Ltd |
| PBR-yenl | pBR322 carrying yenl ORF, Amp<sup>+</sup> | This study |
| pKD3 | Cm<sup>+</sup>; Cm cassette teplate | Stored in this lab |
| pKD46 | Amp<sup>+</sup>, λRed recombinase | Stored in this lab |
| pCP20 | Amp<sup>+</sup>, Cm<sup>+</sup>; Flp recombinase | Stored in this lab |
as the reference APEC strain. Avian macrophage-like cell line HD11 and chicken embryo fibroblast cell line DF-1 were cultivated in DMEM with 10% FBS (37 °C, 5% CO₂). The bioluminescence reporter E. coli pSB401 strain used as an AHL biosensor was cultured in LB at 37 °C. C6HSL was purchased from Santa Cruz Biotechnolog (SC-205405; Dallas, TX, US). SPF chicken sera were purchased from Nanjing Huauen Company (Nanjing, China).

Construction of recombinant strains

The CE129 sdiA gene in-frame deletion mutant, CE129ΔsdiA, was constructed by a Red-based recombination system (Datsenko and Wanner 2000) using RsdiA-up and RsdiA-lo primers. The sdiA open-reading frame (ORF) was amplified using the primer pair, sdiA-1/sdiA-2 (Table 2). The complement plasmid pBR-sdiA was constructed and transformed into CE129ΔsdiA to restore sdiA expression in the mutant strain. The pBR-yenI plasmid was obtained from a previous study, which conferred the E. coli strain endogenous AHL synthesis ability and was transformed into CE129 to obtain CE129/pyenI. Additionally, the pBR322 plasmid was transformed into CE129 to construct the negative control strain. The resulting recombinant CE129/pyenI strain produced AHLs and was measured in bioluminescence assays with the biosensor pSB401, which differed from wild-type CE129 and the negative control CE129/pBR. Overnight cultures of pSB401 were diluted to 1:100 in 1 mL fresh LB medium, and then, 100 µL bacterial cultures were added. After incubation for 6 h, bioluminescence was measured using a Tecan GENios Plus microplate reader in luminescence mode (Tecan Deutschland GmbH, Salzburg, Austria) (Yang et al. 2018a, b). The data were expressed as relative light units of luminescence and were obtained from each sample.

Evaluation of bacterial growth rate

CE129, CE129 with exogenous C6HSL signal (100 µM), CE129/pyenI, CE129/pBR, CE129AsdiA, and CE129AsdiA/sdiA were cultured at 37 °C in LB medium. Culture aliquots were obtained at various time points for absorbance measurements (OD₆₀₀) (Duan et al. 2013).

Serum bactericidal assays

Serum bactericidal assays were performed as previously described (Palaniyandi et al. 2013). Briefly, sera from SPF chickens were collected and diluted with PBS (pH 7.2) in 0.5, 2.5, 5, 12.5, and 25% serum solution. Then, 25% inactivated serum (56 °C, 30 min) solution was prepared, and 10 µL of each strain (10⁶ colony forming units (CFU) of CE129, CE129/pyenI, CE129/pBR, CE129 with 100 µM C6HSL, CE129ΔsdiA with 100 µM C6HSL, and CE129ΔsdiA/sdiA with 100 µM C6HSL) was added to 190 µL serum suspension and disabling serum, and then incubated at 37 °C for 30 min. Viable-cell counts were determined by serially diluting and plating cultures on LB agar at 0, 1, and 2 h after incubating the serum.

Acid resistance (AR) assays

Bacteria were grown overnight in LB at 37 °C and diluted in fresh LB to the early stationary phase (OD₆₀₀ = 2.0). Cultures were centrifuged at 5000 rpm and incubated at acidic pH 2.5 containing 1.5 mM glutamate (Yang et al. 2018a, b). Cells were incubated at 37 °C without shaking. Viable-cell counts were determined by serially diluting and plating cultures on LB agar for 1 and 2 h after the acid challenge.

Competitive growth assays in vitro

CE129ΔsdiA, CE129, CE129/pyenI, and CE129/pBR were grown overnight in LB at 37°C, and then diluted in fresh LB (OD₆₀₀ = 1.0). The same amount of two strains (Group 1: CE129ΔsdiA vs CE129/pyenI; Group 2: CE129ΔsdiA vs CE129/pBR with 100 µM C6HSL; Group 3: CE129ΔsdiA vs CE129/pBR) was added to 5 mL LB. Exogenous 100 µM C6HSL was mixed in LB with Group 2. Bacterial counts were determined at 0, 3, 6, and 9 h after co-incubation by serially diluting and plating cultures on untreated LB agar plates or LB agar plates containing 100 mg/L ampicillin.

Table 2 Primers used in this study

| Primer | Sequences (5’-3’) |
|--------|------------------|
| sdiA-up | 5’-GCCTGAGCTGCTTAGTGAAT-3’ |
| sdiA-lo | 5’-GGCCTAAGGAACACAGTACGG-3’ |
| RsdiA-up | 5’-GATCCCGATACGATTACGG-3’ |
| RsdiA-lo | 5’-GAGTGCCTGCTAGTGAAT-3’ |
| sdiA-RT-F | GCCTGAGCTGCTTAGTGAAT |
| sdiA-RT-R | CCCACGCTTCAGGTAAT |
| gapA-RT-F | 5’-GCCGACGCAACAGAT-3’ |
| gapA-RT-R | 5’-ACGGTGGGACAGACGTC-3’ |
| fimA-RT-F | 5’-ACGGTGGGACAGACGTC-3’ |
| fimA-RT-R | 5’-ACGGTGGGACAGACGTC-3’ |
| fliC-RT-F | 5’-TGACAAAGGGCCGTCAGTT-3’ |
| fliC-RT-R | 5’-CGCCGACCTGGGACAGACGTC-3’ |
| iss-RT-F | 5’-AATTTTGTGCGCGGAT-3’ |
| iss-RT-R | 5’-CAGCGGAGTATAGTATGCGGTAAC-3’ |
Chicken macrophage engulfment and survival assays

Engulfment and survival assays were performed using HD11 previously described (Han et al. 2013; Palaniyandi et al. 2013; Yang et al. 2014). The cell monolayer was co-incubated with $10^7$ CFU bacteria for 2 h, gently washed three times with PBS, and supplemented with 140 mg/mL gentamicin in culture medium for an additional 4 h to kill extracellular bacteria. After Triton X-110 treatment, the number of bacteria that engulfed HD-11 cells was enumerated.

Bacterial adherence assays

The adherence of strains to DF1 cells was determined using a quantitative adhesion assay (Scaletsky et al. 1984; Duan et al. 2012). Briefly, $10^7$ CFU of bacteria was added to each well of a 96-well tissue culture plate with a monolayer of approximately $10^5$ DF-1 cells. After incubation for 1 h, the cell monolayer was gently washed with PBS (pH 7.2) and lysed with 0.5% Triton X-100 for 20 min. Triton X-100 lysates containing total cell-associated bacteria were diluted to 1:10 in PBS and plated on an LB agar plate for bacterial counting (Duan et al. 2012).

Motility assays

Bacteria were seeded onto the center of 0.3% agar plates (1% tryptone, 0.25% NaCl, and 0.3% agar). Motility halos were measured in each strain as previously described (Sperandio et al. 2010; Yang et al. 2013).

Biofilm formation assays

Strains were seeded on biofilm-inducing media (10 g/L tryptone, 5.0 g/L yeast extract, 2.5 g/L NaCl, 3.0 g/L KH₂PO₄, 7.0 g/L K₂HPO₄, 2.0 g/L (NH₄)₂SO₄, 0.5 mg/L FeSO₄, 1.0 g/L MgSO₄, and 2.0 g/L thiamine hydrochloride) in 96-well plates as previously described (Zhou et al. 2020). The OD₆₀₀ values of each well were recorded to measure the amount of biofilm production using a crystal violet staining method. Each strain was tested using six replicates, and the experiment was conducted in triplicate (Yang et al. 2013). To observe biofilm morphologies, sterilized coverslips (2.5 cm × 2.5 cm) were added to 6-well plates, and strains were added to each well at a concentration of $10^5$ CFU/mL. After crystal violet staining and PBS washing, microscopic observations were performed.

Measurement of mRNA levels

A Tiangen RNA Extraction Kit (DP419) was used to prepare total RNA from each strain (Yang et al. 2013). Primers for RT-qPCR assays of the flic, fimA, gadA, and iss genes were designed and are listed in Table 2. Gene gapA was used as the endogenous reference. Fluorescence quantitative RT-qPCR amplification was carried out using SYBR® Premix Ex Taq II (Takara, Shiga, Japan). Assays were conducted in a one-step reaction using an ABI 7500 Real Time System (Applied Biosystems). Three replicates were used in each experiment. All data were normalized to the endogenous reference gene, gapA, using the $2^{-\Delta\Delta CT}$ method.

Statistical analyses

All experiments were conducted in triplicate. Data are presented as the mean ± standard deviation. To evaluate significant differences, Student’s t test method was employed with a significance threshold of $p < 0.05$.

Results

Recombinant strains verified with bioluminescence and RT-qPCR

E. coli cannot synthesize AHL due to lack of the AHL synthase gene, luxI (Smith et al. 2011); thus, previous studies have generally transferred luxI or its homologues from other bacteria into E. coli to construct AHL-producing strains. In this study, yenI (the AHL-encoding gene in Y. enterocolitica) was transformed into APEC CE129 to confer AHL production, which was verified by the reporter strain, pSB401, and was used for short-side chain AHL detection. In the biofilm assays (Fig. 1A), culture supernatants from wild-type CE129 and CE129/pBR failed to induce bioluminescence in pSB401, which differed from the CE129/pyenl recombinant strain. CE129ΔsdiA was also constructed from the parent strain CE129. A large decline of sdiA mRNA expression levels was observed in CE129ΔsdiA (Fig. 1B). Complementation of CE129ΔsdiA with pBR-sdiA restored the mRNA expression levels of sdiA to some extent. Wild-type CE129, CE129/pyenl, and CE129/pBR were used to analyze the function of endogenous AHL, which was secreted by the APEC recombinant strain, CE129/pyenl. Wild-type CE129, CE129ΔsdiA, and CE129ΔsdiAsdiA were applied in exogenous AHL function exploration assays, in which commercial C6HSL was added to bacterial medium to simulate quorum eavesdropping. Growth curves were plotted to assess the growth rates of CE129, CE129/pyenl, CE129/pBR, and CE129 (100 µM C6HSL), CE129ΔsdiA (100 µM C6HSL), and CE129ΔsdiAsdiA (100 µM C6HSL) (data not shown). No significant differences were observed among the growth periods of these six strains (data not shown).
Endogenous and exogenous AHL affected APEC CE129 survival characteristics

In the serum bactericidal assays, there were no significant differences detected among the three strains of endogenous AHL (CE129, CE129/pyenI, and CE129/pBR) or exogenous AHL (CE129 + 100 µM C6HSL, CE129ΔsdiA + 100 µM C6HSL, and CE129ΔsdiA/sdiA + 100 µM C6HSL), which was consistent with the RT-qPCR assay of the serum survival iss gene (data not shown).

Wild-type APEC CE129 and recombination strains were employed to check whether the AR of CE129 was also affected by endogenous AHL (CE129, CE129/pyenI, and CE129/pBR) or exogenous AHL (CE129 + 100 µM C6HSL, CE129ΔsdiA + 100 µM C6HSL, and CE129ΔsdiA/sdiA + 100 µM C6HSL) (Fig. 2A). No significant differences were detected between AHL treated and untreated strains, no matter they were endogenous or exogenous when we tested them.

To identify differences between the APEC and enteropathogenic E. coli strains, or determine if the differences were derived from the special genetic characteristics of CE129 itself, three additional APEC strains were used for the AR assays under AHL influence. These three strains belonged to the three dominant serotypes of the APEC strains from China (O78 serotype strain CE2, O1 serotype strain CE11, and O2 serotype strain CE12). Enterohemorrhagic E. coli (EHEC) O157/H7 86-24 and enterotoxigenic E. coli (ETEC) F18 107/86 were used as the positive controls in the AR assays. In the four APEC strains tested, AR was not obviously different under 100 µM exogenous C6HSL; however, more viable bacteria were observed in the EHEC O157/H7 86-24 and ETEC F18 107/86 groups under acidic pressure, which increased by 3.1- and 2.6-fold, respectively (Fig. 2B).

gadA is one of the key genes that E. coli uses to exercise AR effects. The fluorescence RT-qPCR method was applied to test the mRNA expression levels of gadA in four APEC strains; EHEC O157/H7 86–24 and ETEC 107/86 were also tested as positive controls (Fig. 2C). The results were consistent with the AR experiments (Fig. 2B), in which there were no significant differences among the gadA expression levels of the AHL-influenced APEC strain and strains cultured without C6HSL.

To test the competitive viability of each strain, three groups of two strains were arranged: Group 1, CE129ΔsdiA vs CE129/pyenI (Amp +); Group 2, CE129ΔsdiA vs CE129/pBR (Amp +) with 100 µM C6HSL; Group 3, CE129ΔsdiA vs CE129/pBR (Amp +). LB agar and LB (Amp +) agar plates were employed for the viable bacteria counts of the two strains in each group. Both endogenous AHL (Group 1) and exogenous AHL (Group 2) significantly suppressed bacterial growth after 9 h of co-incubation by 30% and 28% (p < 0.05), respectively, compared to CE129ΔsdiA, which lost AHL perception. In Group 3, without the AHL effect, CE129ΔsdiA did not gain a growth advantage over CE129/pBR (Fig. 2D).

Endogenous AHL improved bacterial survival within the chicken macrophage cell line, HD11. Engulfment and survival assays were performed using HD11. An 83% increase
in survival was observed in CE129/pyenI compared to wild type and CE129/pBR (Fig. 2E), indicating that endogenous AHL upregulated the survival ability of CE129 in chicken macrophage cells. A previous study found that AHL signals directly affected different kinds of cells; therefore, the exogenous AHL group was not arranged in the engulfment or adherence assays, in which only HD11 and DF1 cells were applied.

![Fig. 2](image_url) A: AHL affected the survival ability of CE129. Acid tolerance was quantified by enumerating bacterial survival as a function of time after seed growth in acidified LB (pH 2.5), which was supplemented with 1.0 mM glutamate. Y-axes indicate the ratio of average recovered CFU and seeded CFU values at the beginning of the AR assays. ** indicates significantly different survival ratios at pH 2.5 (p < 0.01). B: Relative gadA transcript abundance comparisons of each strain, which were untreated by C6HSL, are plotted. * indicates significantly different gene expression levels compared to AHL untreated strains (p < 0.05). C: Competitive growth assays. Y-axes indicate the ratio of average CFU values of CE129/pyenI, CE129/pBR, and CE129/pBR compared to CE129△sdiA. * indicates significantly different survival ratios of competitive growth (p < 0.05). D: Quantification of the survival CFU of CE129, CE129/pyenI, and CE129/pBR recovered from HD11 in the engulfment and survival assays. Y-axes indicate averaged CFU values recovered from each well of a 96-well plate. ** indicates significantly different CFU recovered values (p < 0.01).

### AHL affected the adherence of APEC CE129

A 66% increase in adherence was observed in CE129/pyenI compared to CE129 and CE129/pBR (Fig. 3A). Flagella and type I fimbriae are two important adhesion factors of APEC. Motility assays were employed to explore whether flagella expression changed. Previous studies showed that AHL inhibits flagella expression in different
strains of enteropathogenic *E. coli*; therefore, a similar phenomenon was expected in this study. Interestingly, in the motility assays, flagella expression levels of CE129 were not affected by AHL (Fig. 3B). RT-qPCR was used to examine endogenous and exogenous AHL influence on flagella (*fliC*) and type I fimbriae (*fimA*) (Fig. 3C). No significant differences were detected in the *fliC* mRNA expression levels, which was consistent with the motility assay results. While type I fimbriae *fimA* was not affected by AHL or the exogenous AHL group, CE129 showed a 30% decline in *fimA* expression.

To clarify whether a difference in flagella expression exists between APEC and enteropathogenic *E. coli* strains, or determine if the difference is derived from the special genetic characteristics of CE129 itself, three additional APEC strains were used in the *fliC* mRNA expression level detection RT-qPCR assays (Fig. 3D). Flagella expression levels in four APEC strains were not suppressed by AHL signals and decreased by 75 and 69% in the EHEC and ETEC strains, respectively.

**Influence of AHL in CE129 biofilm formation**

The ability of APEC strains to form biofilms was also determined. The biofilm in the 96-well microtiter plate was quantified by optical absorbance at OD$_{600}$ (nm) after solubilizing with ethanol. CE129/pyenI exhibited ~35% of the wild-type and CE129/pBR absorbance in the endogenous AHL group, which was consistent with a 70% decrease of biofilm in CE129 and CE129△sdiA/sdiA in the exogenous AHL group (Fig. 4A). To observe biofilm morphology, sterilized coverslips (2.5 cm×2.5 cm) were added to the 6-well plates. After crystal violet staining and PBS washing, the microscopic observation was performed (Fig. 4B). A similar phenomenon was confirmed compared to the 96-well microtiter plate data (Fig. 4A), indicating the suppression effects of AHL on CE129 biofilm formation.
Discussion

To examine QS-1 effects on APEC CE129, the strain was complemented with the *Y. enterocolitica*-derived *yenI* gene to endogenously produce AHLs (Atkinson et al. 2006). Additionally, the AHL receptor, *sidA*, was deleted in CE129 to form CE129△*sidA*, which lacks AHL detection ability. Construction of recombinant strains was confirmed by pSB401 bioluminescence and fluorescence RT-qPCR assays (Fig. 1). Both exogenous and endogenous AHL did not influence the growth curves of CE129, ensuring the feasibility of subsequent assays.

Changes in the possibly survival ability of CE129 under AHL were subsequently discussed, based on recombinant strains. The serum is a complement-sufficient bactericidal environment (Palaniyandi et al. 2013), and serum resistance
in APEC is linked to different genes, such as *iss*. Both exogenous and endogenous AHL did not participate in the regulation of serum resistance or *iss* expression in CE129.

We previously found that EHEC O157/H7 86–24 and ETEC F18 107/86, which endogenously synthesize AHL, exhibited significantly enhanced AR responses (Yang et al. 2018a, b). Robust AR is a classic feature of enterobacterium strains, by which strains are able to pass through acidic stomach environments and play pathogenic roles at low doses (Van Houdt et al. 2006). The results of this study were consistent with the findings of other papers that discuss the relationship between QS and the AR system in enteropathogenic *E. coli*. Moreover, it has not been clarified whether QS in APEC also affects acid-tolerant systems. Interestingly, the phenomenon that *E. coli* AR is regulated by AHL was not observed in APEC CE129, which differed considerably from ETEC and EHEC (Fig. 2A). To clarify whether a difference exists between APEC and enteropathogenic *E. coli* strains, or determine if the difference is derived from special genetic characteristics of CE129 itself, three APEC strains were used for the AR test with APEC under AHL influence. The three strains belong to the three dominant serotypes of APEC strains in most poultry farms in China (O78 serotype strain CE2, O1 serotype strain CE11, and O2 serotype strain CE12). In all four APEC strains, AR did not obviously change under 100 µM exogenous C6HSL, while more viable bacteria were observed in EHEC O157/H7 86–24 and ETEC 107/86 under acidic pressure, which increased by 3.1- and 2.6-fold, respectively (Fig. 2B). These results were consistent with the *gadA* mRNA expression experiments (Fig. 2C). *gadA* is one of the key genes by which *E. coli* exercises AR effects and has been reported to participate in the QS-induced AR upregulation of ETEC and HEC. APEC infects the avian respiratory tract and first colonizes the air sacs and has different infection routes for HEC and ETEC. It is not necessary to survive in the acidic environment of the digestive tract; therefore, its AR may exhibit different AHL regulation characteristics compared to enteropathogenic *E. coli*.

The growth curve assay revealed that there were no differences in the growth conditions between wild-type CE129 and the recombinant strain. However, the growth curve assay was conducted using individual cultivation conditions and the assumption that the APEC strain may possess a competitive growth advantage under AHL needed to be verified. Because CE129*ΔsdiA* cannot perceive AHL signals, competitive viability assays between two different strains were targeted. The same amount of CE129*ΔsdiA* was added to CE129/*pyenl*, CE129/*pBR*, and CE129/*pBR* with 100 µM C6HSL for co-incubation. After 9 h, CE129/*pyenl* exhibited a clear disadvantage and decreased by 30% in the competitive environment, whereas CE129*ΔsdiA* and CE129/*pyenl* co-incubated, indicating that endogenous AHL inhibited the competitive growth of APEC. Additionally, CE129/*pBR* also exhibited a disadvantage and decreased by 28% in the competitive environment, whereas CE129*ΔsdiA* and CE129/*pBR* co-incubated with 100 µM C6HSL, indicating that exogenous AHL also induced competitive growth disadvantages. AHL stimulation may activate some potential pathways of the APEC strain and consume energy required for its growth, resulting in a competitive growth disadvantage. This growth disadvantage is reflected in the planktonic and extracellular growth states of APEC, which may have possible implications in the survival ability of bacteria in a growth or biofilm state.

In the process of APEC infection in avian respiratory tracts, macrophages play an important antibacterial role with leukocytes. Whether APEC can evade or resist macrophages is crucial for APEC virulence. In the engulfment and survival assays, an 83% increase in survival was observed for CE129/*pyenl* compared to wild type and CE129/*pBR* (Fig. 2E), indicating that endogenous AHL upregulated the survival ability of CE129 in chicken macrophage cells. Bacteria need to evolve to evade and resist the killing activity of macrophages, which is important for its virulence. When CE129 is co-infected with AHL-positive strains in the host, pathways activated by AHL may weaken the competitive growth of extracellular bacteria. Additionally, it may also upregulate the intracellular viability of bacteria, obtaining an intracellular survival advantage from quorum eavesdropping.

In the DF-1 adherence assay, adherence of CE129/*pyenl* increased by 66% compared to CE129 and CE129/*pBR* (Fig. 3A). Subsequently, this study attempted to target the main adhesin that mediates this change in adherence under the influence of AHL. Flagella and type I fimbriae are two important adhesion factors in APEC. Therefore, motility assays were employed to explore whether flagella expression levels changed (Fig. 3B). RT-qPCR was also employed to examine the mRNA expression levels of the flagella gene, *fliC*, and the type I fimbriae gene, *fimA* (Fig. 3C). Results revealed that the increase in adherence did not come from flagella or type I fimbriae, indicating that there may be other adhesins being upregulated by AHL, which requires further investigation. Flagella are important bacterial virulence factors that provide bacterial motility and contribute to the bacterial colonization of host cells and penetration of the mucosal layer. It has been proposed that flagella allow enteric bacteria to exploit inflammation to compete with intestinal microbiota in vivo (Stecher et al. 2004, 2008; Duan et al. 2013; Yang et al. 2013). Previous studies revealed a close relationship between QS and *E. coli* flagella expression, in which flagella expression is inhibited by AHL in several *E. coli* strains. The flagella expression of three APEC strains was therefore examined, and similar results were obtained (Fig. 3D), indicating that AHL regulated flagella expression in APEC is considerably different.
from enteropathogenic *E. coli*. This phenomenon may be derived from the infection route differences between APEC and enteropathogenic *E. coli* strains; however, the mechanism remains to be elucidated.

Biofilms are communities of microorganisms that have attached to a solid surface through extracellular polymeric substances and are related to many essential virulence factors that contribute to colonization, immune escape, and antibiotic resistance (Yang et al. 2013, 2014). AHL elicited CE129 disadvantages in the planktonic growth state, but it brought advantages in the intracellular growth state, and CE129 biofilm formation under AHL influence was subsequently examined. Results revealed that both exogenous and endogenous AHL suppressed biofilm formation in polystyrene 96-well plates (Fig. 4A) and glass surfaces (Fig. 4B).

In conclusion, this study demonstrated that AHL results in extracellular growth disadvantages in APEC CE129 in both a planktonic and biofilm state. AHL also upregulated the intracellular survival ability of CE129 in macrophage cells. AHL exerts an advantage on CE129 adherence, which is the first step in APEC infection and plays a key role in its virulence. Thus, for the first time, this study uncovered the differences between APEC and enteropathogenic *E. coli* strains in terms of AHL function upon flagella expression and AR. Earlier studies found that AHL inhibited the expression of *E. coli* flagella and increased AR, but similar phenotypes did not exist in APEC. This difference may be derived from the different infection routes of APEC and enteropathogenic *E. coli*; however, the mechanism requires further investigation. This study also provides a new perspective on the function of QS-1 in the regulation of APEC strains.

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Author contributions ZG and YY conceived and designed the experiments. XZ, BZ, ZL, QD, and ZM performed experiments. YY and XZ wrote this paper.

Declarations

Conflict of interest The authors and their institutions do not have any relationships that may influence or bias the results and data presented in this manuscript.

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