# Quorum Sensing-1 Signaling of N-hexanoyl-L-Homoserine Lactone Contributes to Virulence in Avian Pathogenic Escherichia Coli

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# Abstract

Avian pathogenic *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, *E. coli* encodes a single LuxR homolog, i.e SdiA, but does not express the LuxI homolog, an acyl-homoserine lactone (AHL) synthase of producing AI-1. Avian pathogenic *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 *Yersinia enterocolitica yenI* gene was expressed into APEC CE129. CE129/p*yenI* was conferred the ability to produce AHL signal. The CE129 SdiA mutant strain with an in-frame *sdiA* (AHL receptor) gene deletion was constructed by a λRed recombination system, which lost the ability to sense AHL.

Aims

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.

Results

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 *E. coli* or enterohemorrhagic *E. coli* (ETEC and EHEC, respectively). These findings enhance our understanding of the QS mechanism.

Conclusions

QS-1 affected APEC biofilm formation, adhesion, and survival ability, but did not affect bacterial acid resistance (AR) or flagella expression, which were observed in ETEC and EHEC. The findings of this study have laid the foundation for further clarifying the complex mechanism of QS in future investigations on the virulence of APEC.

# Introduction

Avian pathogenic *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 process(Yang et al. 2014). 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, sporulation, and biofilm formation(Reading et al. 2006; Walters et al. 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; Yang et al. 2018). 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-1 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; Dyszel et al. 2010; Smith et al. 2011; 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 *yenI* gene from *Yersinia enterocolitica* was subsequently constructed, which was employed to analyze the effect of endogenous synthetic AHL. *yenI* synthesizes AHL signals like C6HSL(Atkinson et al. 2006; Yang et al. 2013; Yang et al. 2018), 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 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% $CO_2$). 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 Biotechnology (SC-205405; Dallas, TX, US). SPF chicken sera were purchased from Nanjing Huaen Company (Nanjing, China).

Table 1
Strains and plasmids used in this study.

| Strain or plasmid | Description | Source or reference |
|---|---|---|
| **Strain** | | |
| 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/p*yenI* | CE129 carrying p*yenI*, Amp$^r$ | This study |
| CE129/pBR | CE129 carrying pBR322, Amp$^r$ | This study |
| CE129△*sdiA* | *sdiA* deficient mutant | This study |
| CE129△*sdiA*/*sdiA* | *sdiA* complement strain | This study |
| ETEC 107/86 | 107/86 carrying p*yenI*, Amp$^r$ | |
| EHEC 8624 | Wild-type EHEC O157:H7 | (Havens et al. 1992) |
| *E. coli* pSB401 | AHL biosensor | Stored in this lab |
| **Plasmid** | | |
| pBR322 | Expression vector, Amp$^r$ | Takara Ltd. |
| pBR-*yenI* | pBR322 carrying *yenI* ORF, Amp$^r$ | This study |
| pKD3 | Cm$^r$; Cm cassette teplate | Stored in this lab |
| pKD46 | Amp$^r$, λRed recombinase | Stored in this lab |
| pCP20 | Amp$^r$,Cm$^r$; Flp recombinase | Stored in this lab |

# Construction of recombinant strains

The CE129 *sdiA* gene in-frame deletion mutant, CE129△*sdiA*, was constructed by a λRed-based recombination system(Datsenko et al. 2000) using R*sdiA*-up and R*sdiA*-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 CE129△*sdiA* 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/p*yenI*. Additionally, the pBR322 plasmid was transformed into CE129 to construct the negative control strain. The resulting recombinant CE129/p*yenI* 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, 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. 2018). The data were expressed as relative light units of luminescence and were obtained from each sample.

Table 2
Primers used in this study.

| Primer | Sequences(5'-3') |
|---|---|
| *sdiA*-up | 5'- CGCCATATGATGCAGGATACGGATT-3' |
| *sdiA*-lo | 5'- GCGCCTAGGAATTAAGCCAGTAGCG-3' |
| R*sdiA*-up | 5'-CCTAAAGTGGCTTTCTACACCAATTACCCTGAGGCGT GGGTTAGTTTGTGTAGGCTGGAGCTGCTTCG-3' |
| R*sdiA*-lo | 5'-CCATAACGGCTGTGCTTCGCTGAATAAGTCAT CATTCCACATTAAACATATGAATATCCTCCTTAG-3' |
| *sdiA*-RT-F | GCGTCGCACGATGCTGTT |
| *sdiA*-RT-R | CCCACGCCTCAGGGTAAT |
| *gapA*-RT-F | 5'- CGTTAAAGGCGCTAACTTCG-3' |
| *gapA*-RT-R | 5'- ACGGTGGTCATCAGACCTTC-3' |
| *fimA*-RT-F | 5'-*GCACAGGAAGGAGCAACCA*-3' |
| *fimA*-RT-R | 5'*GGCAACAGCGGCTTTAGATG*-3' |
| *fliC* -RT-F | 5'- TGACAAAGCCGTCGCAAGTA-3' |
| *fliC*-RT-R | 5'- AGCTGCACCAAAACCAACATC-3' |
| *iss*-RT -F | 5'-AATTTGTGGCGGTGCAGAA-3' |
| *iss*-RT -R | 5'-CAGCGGAGTATAGATGCCAAAA-3' |

# Evaluation of bacterial growth rate

CE129, CE129 with exogenous C6HSL signal (100 μM), CE129/p*yenl*, CE129/pBR, CE129Δ*sdiA*, and CE129Δ*sdiA/sdiA* were cultured at 37°C in LB medium. Culture aliquots were obtained at various time points for absorbance measurements ($OD_{600}$)(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 ($10^6$ colony forming units (CFU) of CE129, CE129/p*yenl*, CE129/pBR, CE129 with 100 μM C6HSL, CE129Δ*sdiA* with 100 μM C6HSL, and CE129Δ*sdiA/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 ($OD_{600}$ = 2.0). Cultures were centrifuged at 5000 rpm and incubated at acidic pH 2.5 containing 1.5 mM glutamate(Yang et al. 2018). 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

CE129△*sdiA*, CE129, CE129/p*yenl*, and CE129/pBR were grown overnight in LB at 37°C, then diluted in fresh LB ($OD_{600}$ = 1.0). The same amount of two strains (Group 1: CE129△*sdiA vs* CE129/p*yenl*; Group 2: CE129△*sdiA vs* CE129/pBR with 100 μM C6SHL; Group 3: CE129△*sdiA vs* CE129/pBR) was added to 5 mL LB. Exogenous 100 μM C6SHL 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.

## 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; Yang et al. 2014). 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. 2002; 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 $KH_2PO4$, 7.0 g/L $K_2HPO4$, 2.0 g/L $(NH_4)_2SO_4$, 0.5 mg/L $FeSO_4$, 1.0 g/L $MgSO_4$, and 2.0 g/L thiamine hydrochloride) in 96-well plates as previously described(Zhou et al. 2020). The $OD_{600}$ 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 $2^{-\triangle\triangle CT}$ 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 bioluminescence assays (Fig. 1A), culture supernatants from wild-type CE129 and CE129/pBR failed to induce bioluminescence in pSB401, which differed from the CE129/p*yenI* recombinant strain. CE129△*sdiA* was also constructed from the parent strain CE129. A large decline of *sdiA* mRNA expression levels was observed in CE129△*sdiA* (Fig. 1B). Complementation of CE129△*sdiA* with pBR-*sdiA* restored the mRNA expression levels of *sdiA* to some extent. Wild-type CE129, CE129/p*yenI*, and CE129/pBR were used to analyze the function of endogenous AHL, which was secreted by the APEC recombinant strain, CE129/p*yenI*. Wild-type CE129, CE129△*sdiA*, and CE129△*sdiA*/*sdiA* 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/p*yenI*, CE129/pBR, and CE129 (100 μM C6HSL), CE129△*sdiA* (100 μM C6HSL), and CE129△*sdiA*/*sdiA* (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/p*yenI*, and CE129/pBR) or exogenous AHL (CE129 + 100 μM C6HSL, CE129△*sdiA* + 100 μM C6HSL, and CE129△*sdiA*/*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/p*yenI*, and CE129/pBR) or exogenous AHL (CE129 + 100 μM C6HSL, CE129△*sdiA* + 100 μM C6HSL, and CE129△*sdiA*/*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, CE129△*sdiA vs* CE129/p*yenI* (Amp+); Group 2, CE129△*sdiA vs* CE129/pBR (Amp+) with 100 μM C6HSL; Group 3, CE129△*sdiA 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 hrs of co-incubation by 30% and 28% ($p <$ 0.05), respectively, compared to CE129△*sdiA*, which lost AHL perception. In Group 3, without the AHL effect, CE129△*sdiA* 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/p*yenI* 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.

# AHL affected the adherence of APEC CE129

A 66% increase in adherence was observed in CE129/p*yenI* 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/p*yenI* 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 *yenl* 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. 2018). 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 EHEC. APEC infects the avian respiratory tract and first colonizes the air sacs and has different infection routes for EHEC 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△sdiA cannot perceive AHL signals, competitive viability assays between two different strains were targeted. The same amount of CE129△sdiA was added to CE129/pyenI, CE129/pBR, and CE129/pBR with 100 μM C6HSL for co-incubation. After 9 hrs, CE129/pyenI exhibited a clear disadvantage and decreased by 30% in the competitive environment, whereas CE129△sdiA and CE129/pyenI 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△sdiA 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/pyenI 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/pyenI 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 fliC 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; Stecher et al. 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; Yang et al. 2014). AHL elicited CE129 disadvantages in the planktonic growth state but it brought advantages in the intracellular growth state, 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.

## Declarations

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

## References

1. Atkinson S, Chang CY, Sockett RE, Camara M, Williams P (2006) Quorum sensing in Yersinia enterocolitica controls swimming and swarming motility. J Bacteriol 188 (4): 1451-61.

2. Boyen F, Eeckhaut V, Van Immerseel F, Pasmans F, Ducatelle R, Haesebrouck F (2009) Quorum sensing in veterinary pathogens: mechanisms, clinical importance and future perspectives. Vet Microbiol 135 (3–4): 187 – 95.

3. Cui Z et al (2016) Autoinducer-2 of quorum sensing is involved in cell damage caused by avian pathogenic Escherichia coli. Microbial Pathogenesis 99: 247–252.

4. 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 (12): 6640-5.

5. Ding X et al (2018) The different roles of hcp1 and hcp2 of the type VI secretion system in Escherichia coli strain CE129. Journal of basic microbiology 58 (11): 938–946.

6. Duan Q et al (2012) The flagella of F18ab Escherichia coli is a virulence factor that contributes to infection in a IPEC-J2 cell model in vitro. Vet Microbiol 160 (1–2): 132 – 40.

7. Duan Q et al (2013) Flagella from F18 + Escherichia coli play a role in adhesion to pig epithelial cell lines. Microb Pathog 55: 32 – 8.

8. Dyszel JL, Soares JA, Swearingen MC, Lindsay A, Smith JN, Ahmer BM (2010) E. coli K-12 and EHEC genes regulated by SdiA. PLoS One 5 (1): e8946.

9. Gao Q, Xia L, Liu J, Wang X, Gao S, Liu X (2016) DNA microarray-mediated transcriptional profiling of avian pathogenic Escherichia coli O2 strain E058 during its infection of chicken. Microbial Pathogenesis 100: 1–9.

10. Gao Q, Xia L, Wang X, Ye Z, Liu J, Gao S (2019) SodA Contributes to the Virulence of Avian Pathogenic Escherichia coli O2 Strain E058 in Experimentally Infected Chickens. JOURNAL OF BACTERIOLOGY 201 (e006256).

11. Han X et al (2013) The luxS gene functions in the pathogenesis of avian pathogenic Escherichia coli. Microb Pathog 55: 21 – 7.

12. Han X et al (2013) The luxS gene functions in the pathogenesis of avian pathogenic Escherichia coli. Microb Pathog 55: 21 – 7.

13. Havens PL, Dunne WM, Burd EM (1992) Effects of human intravenous immune globulin on diarrhea caused by Shiga-like toxin I and Shiga-like toxin II in infant rabbits. Microbiol Immunol 36 (10): 1077-85.

14. Li J, Attila C, Wang L, Wood TK, Valdes JJ, Bentley WE (2007) Quorum sensing in Escherichia coli is signaled by AI-2/LsrR: effects on small RNA and biofilm architecture. J Bacteriol 189 (16): 6011-20.

15. Palaniyandi S, Mitra A, Herren CD, Zhu X, Mukhopadhyay S (2013) LuxS contributes to virulence in avian pathogenic Escherichia coli O78:K80:H9. Vet Microbiol 166 (3–4): 567 – 75.

16. Palaniyandi S, Mitra A, Herren CD, Zhu X, Mukhopadhyay S (2013) LuxS contributes to virulence in avian pathogenic Escherichia coli O78:K80:H9. Vet Microbiol 166 (3–4): 567 – 75.

17. Reading NC, Sperandio V (2006) Quorum sensing: the many languages of bacteria. FEMS Microbiol Lett 254 (1): 1–11.

18. Scaletsky IC, Silva ML, Trabulsi LR (1984) Distinctive patterns of adherence of enteropathogenic Escherichia coli to HeLa cells. Infect Immun 45 (2): 534-6.

19. Smith JL, Fratamico PM, Yan X (2011) Eavesdropping by bacteria: the role of SdiA in Escherichia coli and Salmonella enterica serovar Typhimurium quorum sensing. Foodborne Pathog Dis 8 (2): 169 – 78.

20. Smith JN et al (2008) SdiA, an N-acylhomoserine lactone receptor, becomes active during the transit of Salmonella enterica through the gastrointestinal tract of turtles. PLoS One 3 (7): e2826.

21. Sperandio V, Torres AG, Kaper JB (2002) Quorum sensing Escherichia coli regulators B and C (QseBC): a novel two-component regulatory system involved in the regulation of flagella and motility by quorum sensing in E. coli. Mol Microbiol 43 (3): 809 – 21.

22. Stecher B, Barthel M, Schlumberger MC, Haberli L, Rabsch W, Kremer M, Hardt WD (2008) Motility allows S. Typhimurium to benefit from the mucosal defence. Cell Microbiol 10 (5): 1166-80.

23. Stecher B, Hapfelmeier S, Muller C, Kremer M, Stallmach T, Hardt WD (2004) Flagella and chemotaxis are required for efficient induction of Salmonella enterica serovar Typhimurium colitis in streptomycin-pretreated mice. Infect Immun 72 (7): 4138-50.

24. Van Houdt R, Aertsen A, Moons P, Vanoirbeek K, Michiels CW (2006) N-acyl-L-homoserine lactone signal interception by Escherichia coli. FEMS Microbiol Lett 256 (1): 83 – 9.

25. Walters M, Sperandio V (2006) Quorum sensing in Escherichia coli and Salmonella. Int J Med Microbiol 296 (2–3): 125 – 31.

26. Wu X et al (2017) The role of the ptsI gene on AI-2 internalization and pathogenesis of avian pathogenic Escherichia coli. Microb Pathog 113: 321–329.

27. Yang Y et al (2013) F18ab Escherichia coli flagella expression is regulated by acyl-homoserine lactone and contributes to bacterial virulence. Veterinary Microbiology 165 (3–4): 378–383.

28. Yang Y et al (2014) Quorum-sensing gene luxS regulates flagella expression and Shiga-like toxin production in F18ab Escherichia coli. Can J Microbiol 60 (6): 355 – 61.

29. Yang Y, Liu Y, Zhou M, Zhu G (2018) Both Quorum Sensing (Qs)-I and Ii Systems Regulate Escherichia coli Flagellin Expression. Pakistan Journal of Zoology 50 (5): 1807–1813.

30. Yang Y, Zhou M, Hardwidge PR, Cui H, Zhu G (2018) Isolation and Characterization of N-acyl Homoserine Lactone-Producing Bacteria From Cattle Rumen and Swine Intestines. FRONTIERS IN CELLULAR AND INFECTION MICROBIOLOGY 8 (155).

31. Yu L et al (2018) Autoinducer2 affects trimethoprim-sulfamethoxazole susceptibility in avian pathogenicEscherichia coli dependent on the folate synthesis-associate pathway. MicrobiologyOpen 7 (4): e00582.

32. Zhou M, Duan Q, Yang Y, Zhu G (2020) Use of Fimbrial Rod for F18ab Fimbriae + STEC Colonization to Host Cells. Journal of Visualized Experiments (163).
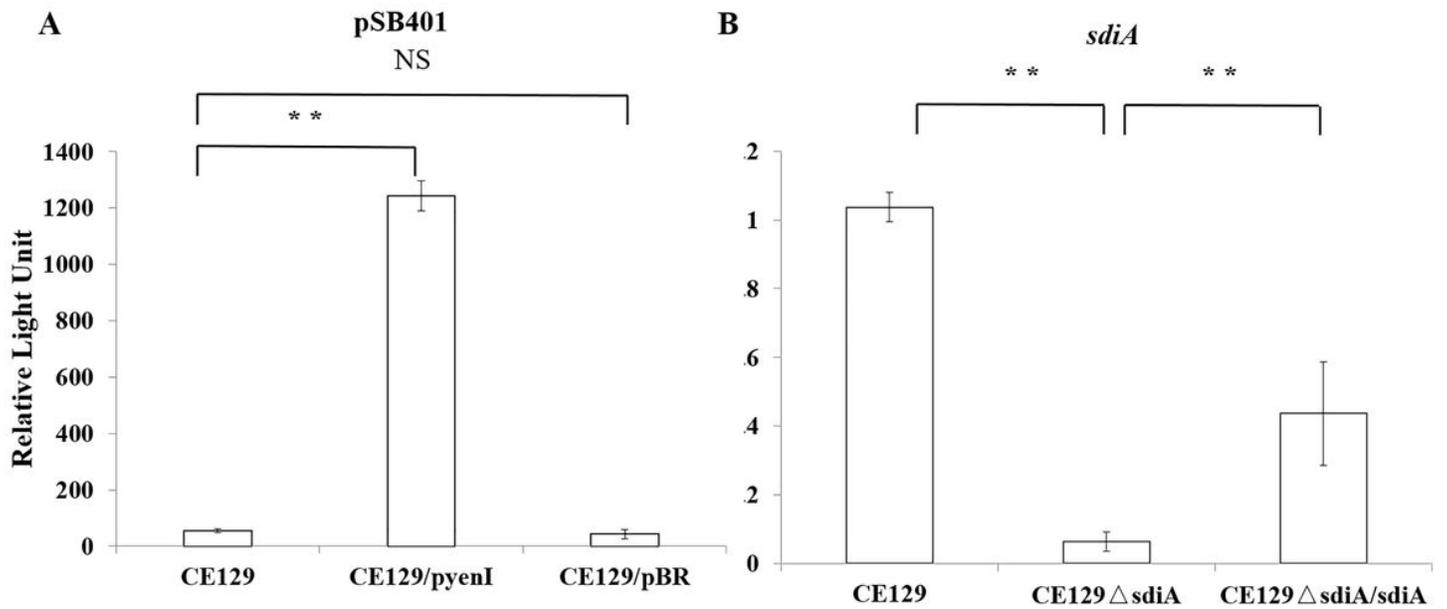
# Figures

**Figure 1**

Confirmation of recombinant strains. A. Bioluminescence (relative light units) of the reporter strain, E. coli pSB401, after the addition of culture supernatants from CE129, CE129/pyenI, and CE129/pBR. AHL induced the bioluminescence of pSB401. ** indicates significantly different relative light units compared to CE129 ($p < 0.01$). B. The relative transcript abundance of sdiA compared to CE129 is plotted. ** indicates significantly different gene expression levels ($p < 0.01$).
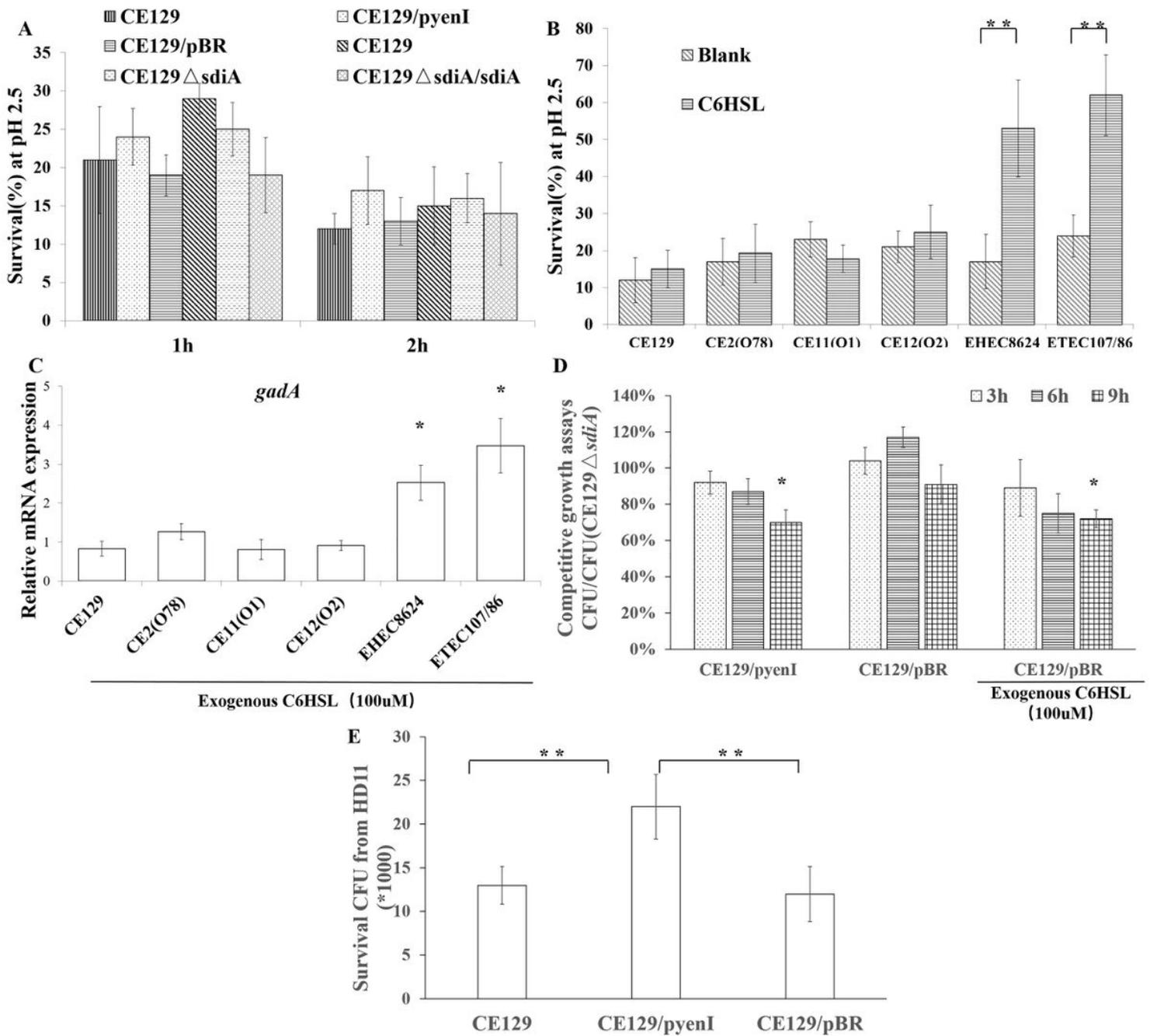
Figure 2

AHL affected the survival ability of CE129. A, B. 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). C. 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). D. Competitive growth assays. Y-axes indicate the ratio of averaged 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). E. 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).
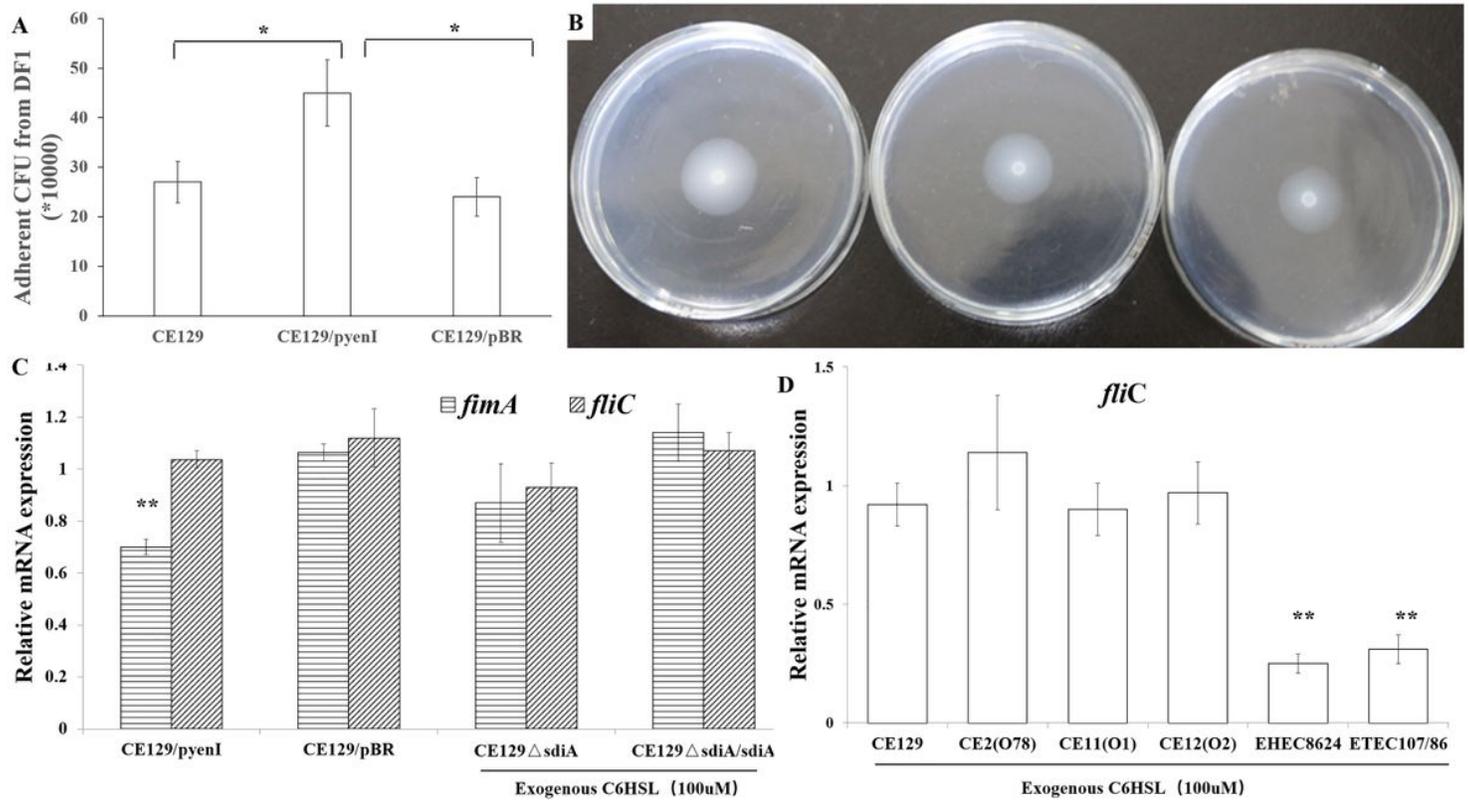


**Figure 3**

AHL influenced the adherence of CE129. A. Quantification of the adherence of CE129, CE129/pyenI, and CE129/pBR compared to DF-1 cells. Y-axes indicate average CFU values recovered from each well of a 96-well plate. * indicates significantly different CFU recovered values (p < 0.05). B. Motility halos were observed and compared to the indicated strains on 0.3% swim agar plates. C. Relative fliC and sdiA transcript abundances compared to CE129 and CE129 with 100 μM in exogenous AHL are plotted. ** indicates significantly different gene expression levels compared to CE129 (p < 0.01). D. Relative fliC transcript abundances compared to each strain, which was not treated by exogenous C6HSL, are plotted. ** indicates significantly different gene expression levels compared to untreated strains (p < 0.01).
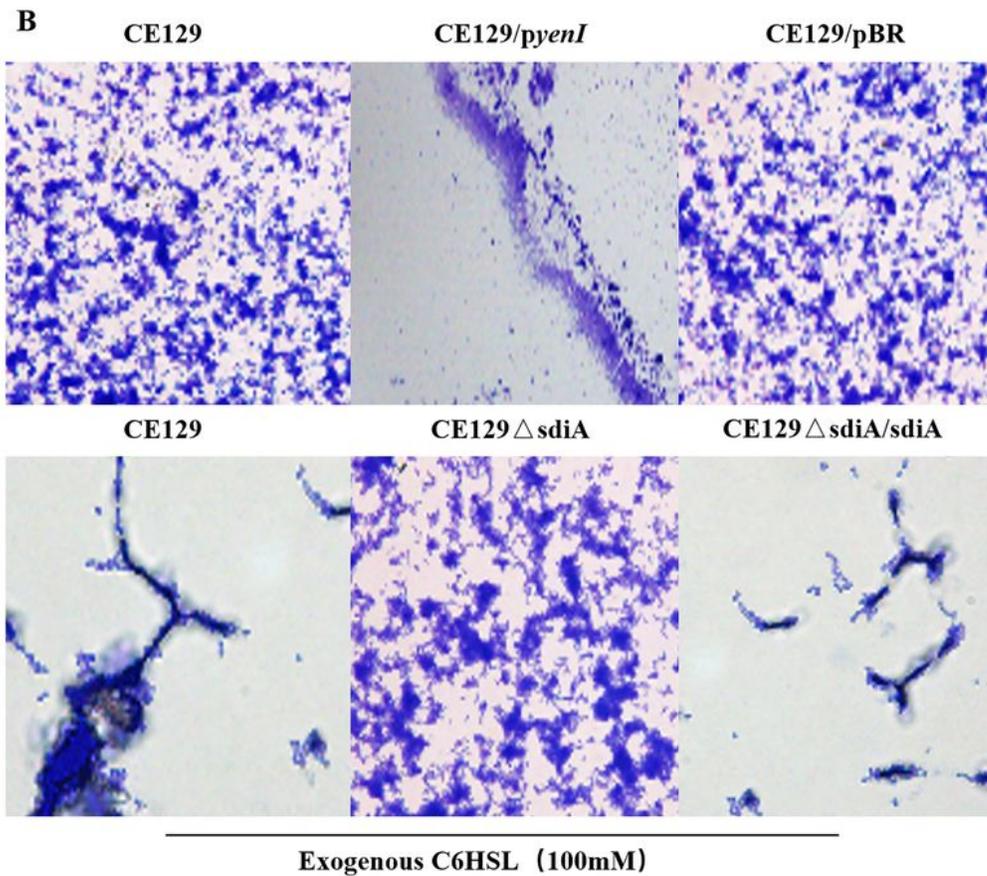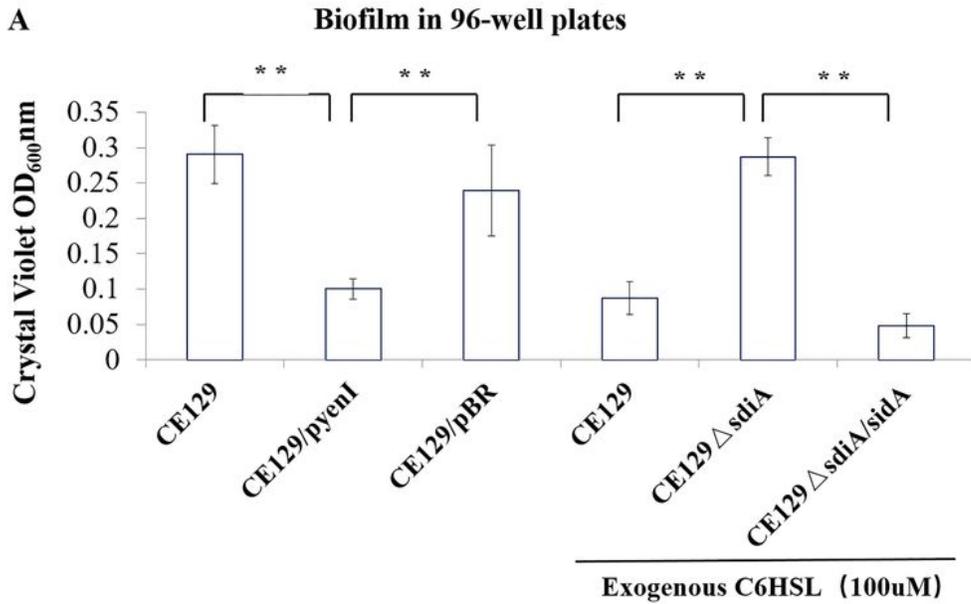
## Figure 4

Effect of AHL on biofilm formation. A. Qualitative analyses of biofilm formation were exerted. Each strain was seeded in biofilm inducing medium. Results of the crystal violet method were observed at OD600. ** indicates significantly different OD600 absorbance values ($p < 0.01$). B. Qualitative analyses of biofilm formation were exerted. Strains were seeded in biofilm inducing medium. Strains of crystal violet were microscopically observed (× 40).