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Both Quorum Sensing (Qs)–I and Ii Systems Regulate *Escherichia coli* Flagellin Expression

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ABSTRACT

For elucidating effect of quorum sensing (QS) systems I and II (QS-1 and QS-II) on *Escherichia coli* flagellin expression, *E. coli* F18ab strain 107/86 was modified to either express acyl-homoserine lactone (AHL) synthase (QS-I) or deleted for autoinducer 2 (AI-2) expression (QS-II). AHL expression and deletion of *luxS* (AI-2) both inhibited flagellin expression, as measured by motility assays, bacterial gene expression, and host responses to infection. The QS systems and flagellin were coordinately regulated, as deleting *fliC* caused decreased QS-II activity.

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Authors' Contribution
GZ designed the study. YY performed experimental work, analyzed the data and wrote the article. YL helped in motility assays. MZ helped in article writing.

Key words
***E.coli*, Flagellin, Quorum sensing-I and II.**

INTRODUCTION

Porcine edema disease and porcine post-weaning diarrhea are two important diseases which bring pigs high morbidity and mortality. Shiga toxin-producing *E. coli* (STEC) is major pathogen of the diseases (Da *et al*., 2001; Frydendahl, 2002). Flagella participate in bacterial pathogenicity as important virulence factor. Besides providing motility, it also contributes to bacterial initial adhesion or colonization to host cells. *In vivo,* enteric bacteria could take advantage of flagella motility to compete with intestinal microbiota by exploiting inflammation (Stecher *et al*., 2004, 2008; Duan *et al*., 2012; Zhou *et al*., 2013).

Quorum sensing (QS) represents one crucial communication system, and was considered as a kind of bacterial-population based language between bacteria (Pacheco and Sperandio, 2009; Curtis and Sperandio, 2011). QS-I positive bacteria normally express acyl-homoserine lactone (AHL) synthase, whereas the QS-II system is regulated by LuxS and autoinducer 2 (AI-2) (Niu *et al*., 2013). We previously reported that QS-I expression in *E. coli* suppresses flagella expression (Yang *et al*., 2013). QS-II also regulates bacterial virulence strategies, including regulate motility by *flhDC*, type III secretion systems (Anand and Griffiths, 2003; Li *et al*., 2007; Han and Lu, 2009), as well as biofilm formation and bacterial pathogenicity (Sperandio *et al*., 2002; Clarke *et al*., 2006; Gonzalez *et al*., 2006).

Here we investigated the extent to which QS-II can also regulate flagellin expression in STEC and examined the potential for coordinate regulation between two QS systems.

MATERIALS AND METHODS

Strains used in this study

Strains and plasmids used are listed in Table I. LB broth or LB agar plates were used for bacterial growth. Caco-2 cell line was cultivated in DMEM with 10 % FBS (37 ˚C, 5 % CO_2). Human TNF and IL-8 immunoassay Kits (R&D Systems, Inc.) were purchased for relative experiments.

Construction of recombinant strains

The F18ab *luxS* gene in-frame deletion mutant (F18abΔ*luxS*) was constructed using λRed-based recombination system (Datsenko and Wanner, 2000). The *luxS* open reading frame (ORF) was amplified by primers *LuxS*-1/ *LuxS*-2 (Table II). Plasmid pBR-*luxS* was constructed and then transformed into F18abΔ*luxS* to obtain the complemented strain F18abΔ*luxS*/p*luxS*.

To over-express *uvrY* and *csrB* in F18ab, the primers *uvrY*-F/*uvrY*-R and *csrB*-F/*csrB*-R were used to PCR amplify *uvrY* and *csrB*, respectively. *uvrY* and *csrB* were cloned into pBR322 and transformed into F18ab *E. coli*.

AI-2 bioassays and motility assays

After grown to an OD600 of 1.3, supernatants of

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strains were collected. Bioluminescence was measured in luminescence mode by Tecan GPM reader (Han and Lu, 2009; Zhou *et al*., 2014). For motility assays, strains were seeded in the middle of motility agar plates. After appropriate growth time, motility halos were measured (Duan *et al*., 2013).

Table I.- Strains and plasmids used in this study.

Strain or plasmid	Description	Source or reference
Strains		
E. coli F18ab 107/86	Wild-type: O139:H1:F18ab, Stx2e; O139:H1:F18ab, Stx2e	Duan *et al*. (2012)
E. coli F18ab/pyenI	107/86 carrying p*yenI*	Yang *et al*. (2013)
E. coli F18ab/pBR	107/86 carrying pBR322	Yang *et al*. (2013)
E. coli F18abΔfliC	*fliC* deletion mutant	Duan *et al*. (2012)
E. coli F18abΔfliC/pfliC	F18abΔ*fliC* carrying pBR-*fliC*	Duan *et al*. (2012)
E. coli F18abΔluxS	*luxS* deletion mutant	This study
E. coli F18abΔluxS/pluxS	F18abΔ*luxS* carrying pBR-*luxS*	This study
E. coli F18ab/pcsrB	107/86 carrying p*csrB*	This study
E. coli F18ab/puvrY	107/86 carrying p*uvrY*	This study
E. coli DH5*a*	AI-2 bioassay negative control	Takara Ltd.
Vibrio harveyi BB170	AI-2 bioassay reporter strain	Bassler *et al*. (1994); Yang *et al*. (2013)
Plasmids		
pBR322	Expression vector, Amp^r	Takara Ltd.
pBR-*luxS*	pBR322 carrying LuxS ORF	This study
pKD3	Cm^r; Cm cassette template	Datsenko and Wanner (2000); Duan *et al*. (2012)
pKD46	Amp^r, λRed recombinase expression	Datsenko and Wanner (2000); Duan *et al*. (2012)
pCP20	Amp^r,Cm^r; Flp recombinase expression	Datsenko and Wanner (2000); Duan *et al*. (2012)

Table II.- Primers used in this study.

Primer	Sequence (5'-3')	Primer	Sequence (5'-3')
LuxS-1	ATGCCGTTGTTAGATAGCTTCAC	*csrA-RT*-F	AGCCTGGATACGCTGGTAGA
LuxS-2	CTAGATGTGCAGTTCCTGCAACT	*csrA-RT*-R	CGAGTTGGTGAGACCCTCAT
Δ*LuxS*-1	TGCAGTTCGGGTGGCGAAAACAATGA ACACCCCGCATGGCGACGCAATCATGT GTAGGCTGGAGCTGCTTCG	*barA-RT*-F	GCTGCGTACACCACTGAATG
		barA-RT-R	TGCGTAGTGGGAATGGAATAC
		fliC-RT –F	CAGCAAGCGGTGAAGTGAA
Δ*LuxS-2*	TCTGATTCTGATCCTGCACTTTCAGCAC GTCTTCCATTGCCGCTTTCCCATATGAAT ATCCTCCTTAG	*fliC-RT*-R	AAGCGTAGCCACAGTAGCA
		flhD-RT-F	ACTGACTCTTCCGCAAATGGT
		flhD-RT-R	TGGCTGTCAAAACGGAAGTG
uvrY-F	TAAGGATCCATGGACATGAGTAT	*il8-RT*-F	TGCAGCTCTGTGTGAAGGTG
uvrY-R	TTAGTCGACTCACTGACTTGATAAT	*il8-RT*-R	ACTTCTCCACAACCCTCTGC
csrB-F	TAAGGATCCGTCGACAGGGAGT	*il10-RT*-F	CCACGCTTTCTAGCTGTTGA
csrB-R	CGCGTCGACAATAAAAAAAGGG	*il10-RT*-R	CTCCGAGACACTGGAAGGTG
gapA-RT-F	CGTTAAAGGCGCTAACTTCG	*tnfa-RT* –F	CCCAGGGACCTCTCTCTAATC
gapA-RT-R	ACGGTGGTCATCAGACCTTC	*tnfa-RT*-R	TGAGGTACAGGCCCTCTGAT
uvrY-RT-F	TACCAGCGAAAGTCATGCAG	GAPDH-*RT*-F	GATGGGCGTGAACCATGAG
uvrY-RT-R	CGTTCAGACAAACTGGCAAA	GAPDH-*RT*-R	GAGGCATTGCTGACGATCTTG

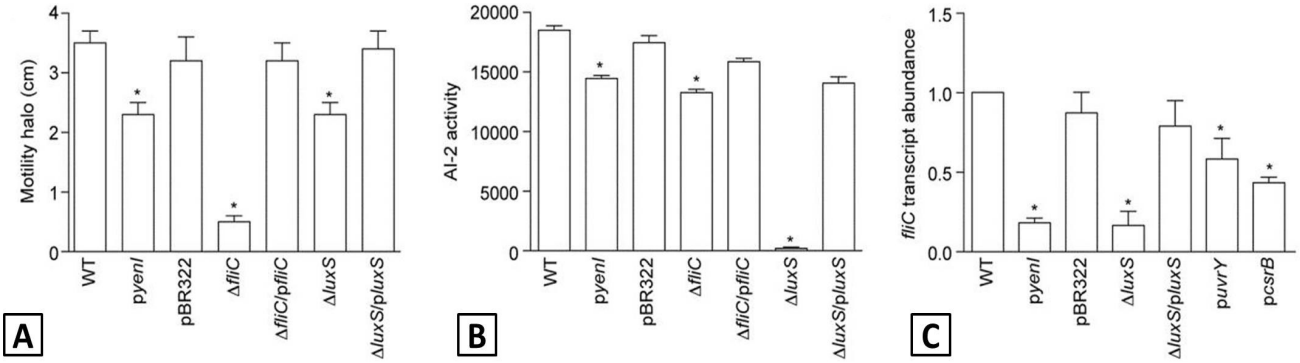

Fig. 1. QS-1 and QS-II have differential impact on bacterial motility. A, motility diameters were quantified after 12 h growth on 0.3 % swim agar plates; B, AI-2 production, AI-2 activity was measured using a bioluminescence assay after growing the indicated bacterial strains to an OD_{600} of 1.3. AI-2 activity is expressed as relative light units measured; C, *fliC* expression. *fliC* expression was measured using qRT-PCR with data normalized to the endogenous reference gene *gapA*. Data are expressed relative to expression in the WT strain.

Measurement of mRNA level

Tiangen RNA Extraction Kit (DP419) was employed in this study, through which total RNA from each strain was prepared (Han and Lu, 2009). Primers for *flhD*, *fliC*, *csrA*, *barA*, *uvrY*, *pfs*, and *luxS* genes were designed and listed in Table II. Gene *gapA* was chosen as the endogenous reference. Caco-2 (1×10^6 cells/well density) was plated in 6-well plates. 10^7 CFU of relative strains were injected into each well, and infected for 2 h. Then cells were dealt with Tiangen RNA Extraction Kit following the standard protocol. $2^{-\Delta\Delta CT}$ method was employed for data analysis.

ELISA assay

Monolayer of Caco-2 was prepared for infection of individual *E. coli* strains. After 2h incubation, supernatants were obtained by centrifugation (Duan *et al*., 2012). With commercial kits, expression levels of IL-8 and TNF were then measured.

Statistics

All experiments were repeated at least 3 times. Data are presented as the mean ± standard deviation. To evaluate statistical significance with $p < 0.05$ considered significant, the Student's t-test method was employed.

RESULTS AND DISCUSSION

QS-1 and QS-II have differential impact on bacterial motility and affect E. coli*-induced pro-inflammatory responses*

F18ab *E. coli* was transformed with the *yenI* gene from *Y. enterocolitica* to induce endogenous AHL production (QS-1) (Yang *et al*., 2013). Flagella involves in bacterial motility, adhesion, invasion. Furthermore, it could induce inflammation response in host cells. With the recombinant strain, function of AHL (QS-1 signals) upon flagella expression could be identified. Subsequently, expressing p*yenI* inhibited bacterial motility on swim agar plates (Fig. 1A). Reduced flagella expression was observed in AHL positive strain F18ab/pyenI, as well as decreased motility ability.

QS-II manipulates multiple genes expression through AI-2. In many pathogenic bacteria, AI-2 participates in regulating bacterial virulence strategies. To assess a similar role for QS-2 on motility, we deleted the *luxS* gene. F18abΔ*luxS* was deficient in AI-2 production (Fig. 1B) and was less motile than the WT strain (Fig. 1A). Consistent with their impaired motility, *fliC* expression was inhibited in both p*yenI* and in Δ*luxS* (Fig. 1C).

Bacterial flagellin can induce pro-inflammatory responses through TLR5. TLR5 stimulates pro-inflammatory genes in NF-κB and MAPK pathways (Vijay-Kumar *et al*., 2010; Salazar-Gonzalez and Navarro-Garcia, 2011). Researchers found that as one pathogen-associated molecular pattern (PAMP), flagellin also have TLR5-independent pro-inflammatory ability by Naip5 and Ipaf, members of the NLR family (Miao *et al*., 2006; Ren *et al*., 2006).

These F18ab *E. coli* strains did not activate the pro-inflammatory IL-8 or TNF responses of infected Caco-2 cells to a magnitude equal to that of infection by WT F18ab *E. coli*, as measured by both RT-PCR (Fig. 2A, B) or by ELISA (Fig. 2C, D). QS-1 expression inhibits F18 *E. coli* motility, whereas QS-II expression enhances motility by affecting *fliC* expression.

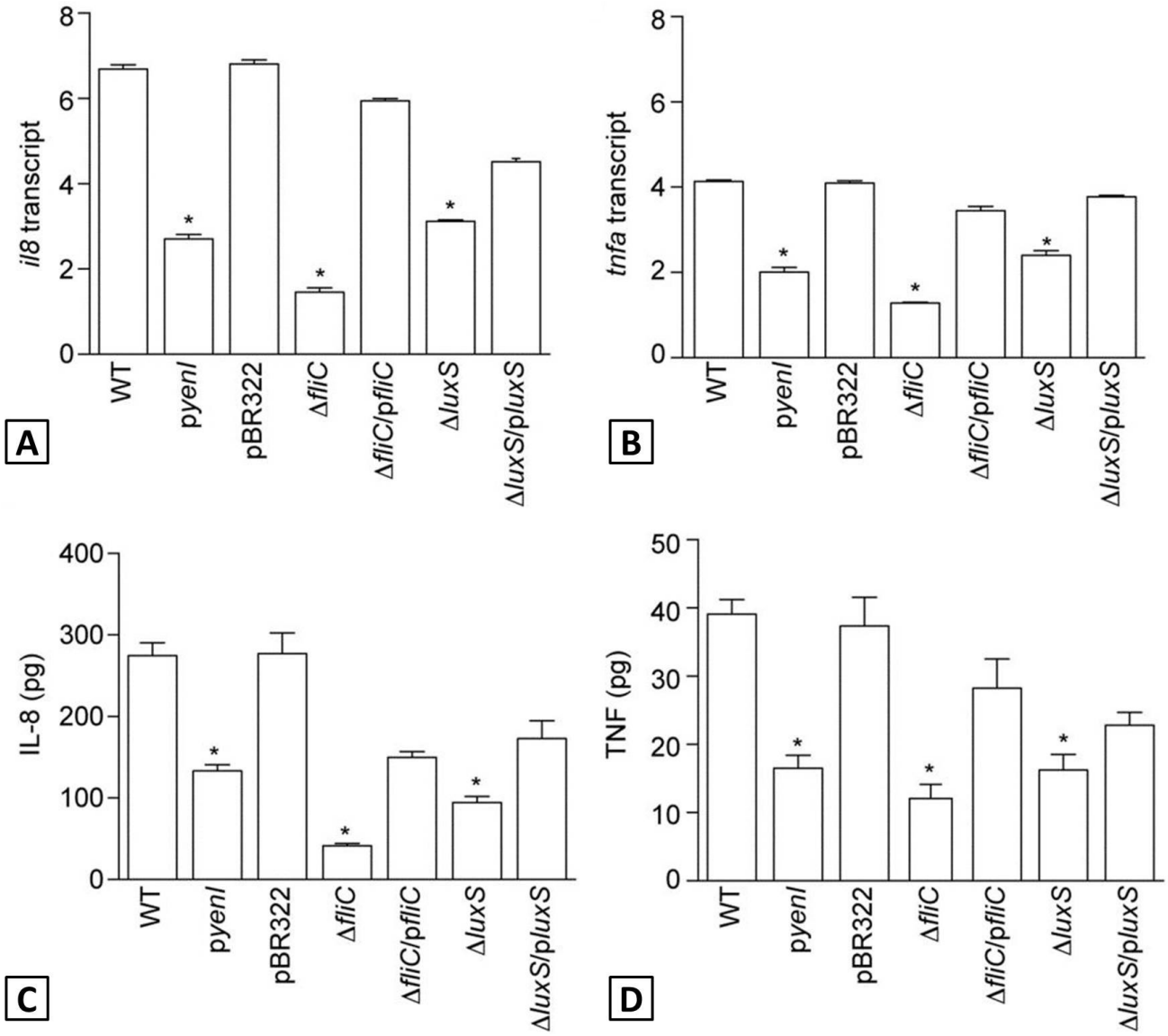

Fig. 2. Host IL-8 and TNF expression in response to F18 *E. coli* infection. A-B, transcription of *il8* and *tnfa* in Caco-2 cells after a 2 h infection with the indicated bacterial strains. Data were normalized to the housekeeping gene *gapdh*; C-D, ELISA, secretion of IL-8 and TNF into Caco-2 cell supernatants after a 2 h infection with the indicated bacterial strains was quantified using ELISAs.

AI-1 and flagella expression influence AI-2

AI-2 activity was impaired in F18ab/pyenI, indicating that QS-1 influences AI-2 production (Fig. 1B). The phenomenon was consistent with the decreased mRNA level of both *luxS* and *pfs* in F18ab/p*yenI* (Fig. 3), which encode enzymes involved in AI-2 synthesis in *E. coli* (Zhu *et al*., 2007). *Pfs*, an important functional enzyme in AI-2 signal synthesis, encounter 25 % decrease of mRNA level under QS-1 influence, while flagella motility was reduced heavily in luxS mutant. FliC expression also regulated QS-II, as deleting *fliC* inhibited *pfs* and *luxS* expression (Fig. 3).

Coordinate regulation of flagella and QS expression

Bacteria utilize two-component systems for adaptation to environmental changes (Pernestig *et al*., 2001, 2003; Herren *et al*., 2006; Yang *et al*., 2014). The BarA/UvrY and the CsrA/CsrB two-component systems regulate flagella expression (Edwards *et al*., 2011). UvrY can activate *barA* expression through an auto-regulatory loop. Influence of CsrA upon *csrB* is regulated partly by *barA*, and a BarA-independent, UvrY-dependent mechanism also involves. CsrA indirectly induces *csrB* transcription. UvrY can directly activate *csrB* transcription, and also is

included in up-regulation of CsrA. Increased transcription of *uvrY* has been observed when *E. coli* is sustained in an AHL-positive environment (Wei *et al*., 2001; Van Houdt *et al*., 2006).

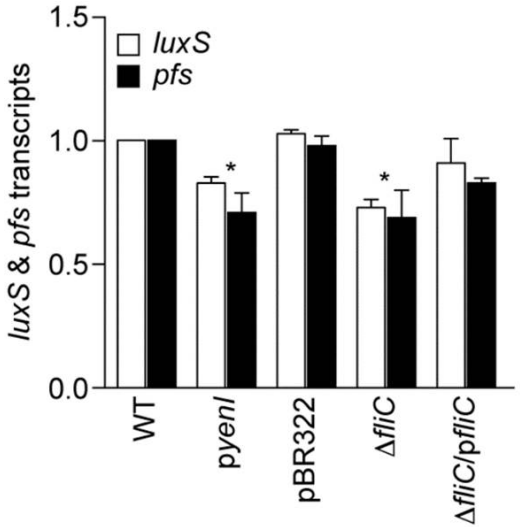

Fig. 3. AI-1 and flagella expression influence AI-2 expression. *luxS* and *pfs* expression were measured using qRT-PCR with data normalized to the endogenous reference gene *gapA*. Data are expressed relative to expression in the WT strain.

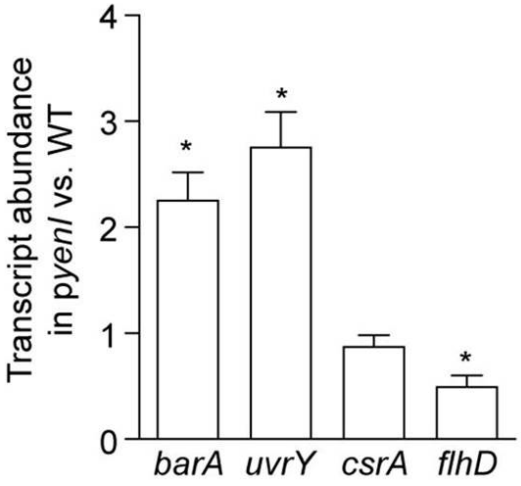

Fig. 4. Coordinate regulation of flagella and QS expression. Transcription of the indicated genes was measured using qRT-PCR. Data are shown as expression in p*yenI* vs. WT.

In this study, expression of *barA* and *uvrY* were increased by 2.3- and 2.7-fold, respectively, in F18ab/p*yenI* (Fig. 4). Up-regulation of *uvrY* and *barA* can induce *csrB* transcription, which binds to CsrA and antagonizes its regulatory effects upon *flhDC* (Liu and Romeo, 1997; Mercante *et al*., 2009; Edwards *et al*., 2011). *flhD* expression did decreased 2.1-fold in p*yenI* (Fig. 4). Over-expressing *uvrY* and *csrB* caused a 1.7- and 2.3-fold reduction in *fliC* expression, respectively (Fig. 1C).

CONCLUSIONS

Overall, we show here that expressing QS-1 in *E. coli* inhibits flagella expression, whereas the LuxS QS-II system up-regulates flagella expression. These systems are coordinately regulated, as QS-I inhibited QS-II and flagellin expression positively regulated LuxS. We implicate the BarA/UvrY and the CsrA/CsrB two-component systems as possible regulators of this coordinated regulation. In the presence of cattle rumen AHLs, *E. coli* O157:H7 represses *LEE* gene expression and activates *gad* expression to improve acid tolerance (Sperandio, 2010; Sheng *et al*., 2013). Other aspects of *E. coli* biology are also regulated by AHLs, including cell division (Sitnikov *et al*., 1996) and antibiotic resistance (Rahmati *et al*., 2002). These data suggest that *E. coli* flagella expression is also regulated by AHLs.

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Statement of conflict of interest

The authors or their institution do not have any relationships that may influence or bias the results and data presented in this manuscript. There is no conflict of interests regarding the publication of the manuscript.

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