

Full Paper

Protozoal ciliate promotes bacterial autoinducer-2 accumulation in mixed culture with *Escherichia coli*

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We have previously demonstrated conjugation of *Escherichia coli* into vacuoles of the protozoal ciliate (*Tetrahymena thermophila*). This indicated a possible role of ciliates in evoking bacterial quorum sensing, directly connecting bacterial survival via accumulation in the ciliate vacuoles. We therefore assessed if ciliates promoted bacterial autoinducer (AI)-2 accumulation with vacuole formation, which controls quorum sensing. *E. coli* AI-2 accumulation was significantly enhanced in the supernatants of a mixed culture of ciliates and bacteria, likely depending on ciliate density rather than bacterial concentration. As expected, AI-2 production was significantly correlated with vacuole formation. The experiment with *E. coli luxS* mutants showed that ciliates failed to enhance bacterial AI-2 accumulation, denying a nonspecific phenomenon. Fluorescence microscopy revealed accumulation of fragmented bacteria in ciliate vacuoles, and, more importantly, expulsion of the vacuoles containing disrupted bacteria into the culture supernatant. There was no increase in the expression of *luxS* (encoding AI-2) or *ydgG* (a transporter for controlling bacterial export of AI-2). We conclude that ciliates promote bacterial AI-2 accumulation in a mixed culture, via accumulation of disrupted bacteria in ciliate vacuoles followed by ex-

pulsion of the vacuoles, independently of *luxS* or *ydgG* gene induction. This is believed to be the first demonstration of a relationship between *E. coli* AI-2 dynamics and ciliates. In the natural environment, ciliate biotopes may provide a survival advantage to bacteria inhabiting such biotopes, via evoking quorum sensing.

Key Words: autoinducer-2; ciliates; *Escherichia coli*; *luxS*; quorum sensing; *Tetrahymena thermophila*; vacuole; *ydgG*

Abbreviations: AI-2, autoinducer-2; CPFX, ciprofloxacin; CTX, cefotaxime; ESBL, extended-spectrum β -lactamase

Introduction

The microbial community ubiquitously seen in natural environments such as soil or pond water comprises a diverse mixture of bacteria and protozoa such as ciliates or amoebae (Novarino et al., 1997; Rodríguez-Zaragoza et al., 1994). While such a microbiota plays a fundamental role in feeding bacteria as a nutrient source for predators such as ciliates or amoebae, the bacteria presumably exploit the microbiota for nutrient-rich environments to ensure survival (Russell and Rychlik, 2001; Tyson et al.,

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Table 1. Bacterial strains and plasmids used for this study.

Bacterial strains and plasmids	Characteristics	Reference
<i>E. coli</i>		
CPF-X-R	Clinical isolate, CPF-X ^r	Oguri et al. (2011)
CTX-M ESBL	Clinical isolate, CTX ^r	Oguri et al. (2011)
CPF-X-R $\Delta luxS$	CPF-X ^r $\Delta luxS$	This study
CTX-M ESBL $\Delta luxS$	CTX-M ESBL $\Delta luxS$	This study
SM10 λ pir	<i>thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu λ pir R6K Km^r</i>	Miller and Mekalanos (1988)
Plasmids		
pYAK1	R6Kori, Cm ^r , <i>sacB</i> ⁺	Kodama et al. (2002)
pYAK1c	pYAK1 cloned $\Delta luxS$ allele of CPF-X-R <i>E. coli</i> strain	This study
pYAK1e	pYAK1 cloned $\Delta luxS$ allele of CTX-M ESBL <i>E. coli</i> strain	This study

Table 2. PCR primers used for this study.

Primer	Sequence (5' > 3')	Reference
For establishment of <i>luxS</i> mutants		
<i>EcluxS</i> -F1	* ggccggatcc [§] CGG CGA AGC GTA TCA GA	This study
<i>EcluxS</i> -R1	tctcttctggcagtgcca CTG CAG GCG CTTC CAT CC	This study
<i>EcluxS</i> -F2	ggatggaagcgcctgcag TGG CAC TGC CGA AAG AGA	This study
<i>EcluxS</i> -R2	ggccgcatgc TTG CCA CCG GAA CGA CTG	This study
<i>Ec luxS</i> -R1-3	ttcttcgttgcgtgtgatgc CTG CAG GCG CTT CCA TCC	This study
<i>EcluxS</i> -F3	ggatggaagcgcctgcag GCA TCA ACA GCA ACG AAG AA	This study
<i>EcluxS</i> -R3	ggccgcatgc ACC GGG TATG GGC AAA GA	This study
<i>EcluxS</i> -CHF	TTG CGG TGT GGC TGG AAA AAC	This study
<i>EcluxS</i> -CHR	GAC GCA ACA GCA GGC ATCA GG	This study
For detection of <i>luxS</i> gene expression		
<i>luxS</i> -F	GAT CAT ACC CGG ATG GAA G	Medellin-Pena et al. (2007)
<i>luxS</i> -R	AGA ATG CTA CGC GCA ATA TC	
For detection of <i>ydgG</i> gene expression		
<i>ydgG</i> -F	CCC TAT CGC TCA GGT ACT GG	This study
<i>ydgG</i> -R	TGT TCA AGC GCA ATT TTG AC	This study
House keeping genes (<i>gst</i> and <i>groEL</i>)		
<i>gst</i> -F	CTT TGC CGT TAA CCC TAA GGG	Mith et al. (2015)
<i>gst</i> -R	GCT GCA ATG TGC TCT AAC CC	
<i>groEL</i> -F	GGN GAC GGN ACN ACN ACN GCA ACN GT	Teng et al. (2001)
<i>groEL</i> -R	TCN CCR AAN CCN GGY GCN TTN ACN GC	

*Bold sequence show linkers for cloning.

[§]Cutting sites of restriction enzymes.

2004), and the acquisition of virulence factors or resistance against unpredictable harmful conditions through gene exchange (Aminov, 2011). Highly packed bacteria, such as in biofilms or ciliate vacuoles, are directly responsible for phenotypic features, possibly with genetic exchanges, against harmful environmental conditions, and also evoke quorum sensing via autoinducer (AI)-2 production (Aminov, 2011; Hojo et al., 2012; Lang and Faure, 2014).

We have previously found that a mixed culture of *Escherichia coli* with *Tetrahymena* ciliates could prove beneficial in gene exchange between the bacteria (Matsuo et al., 2010; Oguri et al., 2011). Since the gene exchanges significantly decline in the presence of phagocytosis inhibitor (latrunculin B), they appear to occur by conjugation via bacterial uptake following accumulation in ciliate vesicles (Matsuo et al., 2010). Thus, our data clearly indicate that ciliate vacuoles provide benefits, via gene exchanges, to the engulfed bacteria. These findings raise

another, fundamental question: whether ciliates promote bacterial quorum sensing via AI-2 accumulation into ciliate vacuoles or to their biotopes. However, it remains unclear.

In the present study, we aimed to verify if ciliates evoke bacterial AI-2 accumulation, using a mixed culture of *Tetrahymena thermophila* and *E. coli* [cefotaxime (CTX)-resistant (CTX-M) extended-spectrum β -lactamase (ESBL) producing and/or ciprofloxacin (CPF-X)-resistant clinical isolates], or *luxS* mutants.

Materials and Methods

Bacteria and protozoa. CTX-resistant ESBL and/or CPF-X-resistant (R) *E. coli* and *T. thermophila* IB were used for the mixed culture. Both bacteria were originally isolated from Hokkaido University Hospital, Japan (Oguri et al., 2011). The bacteria were statically cultured in LB broth containing 1% NaCl, 1% peptone and 0.5% yeast

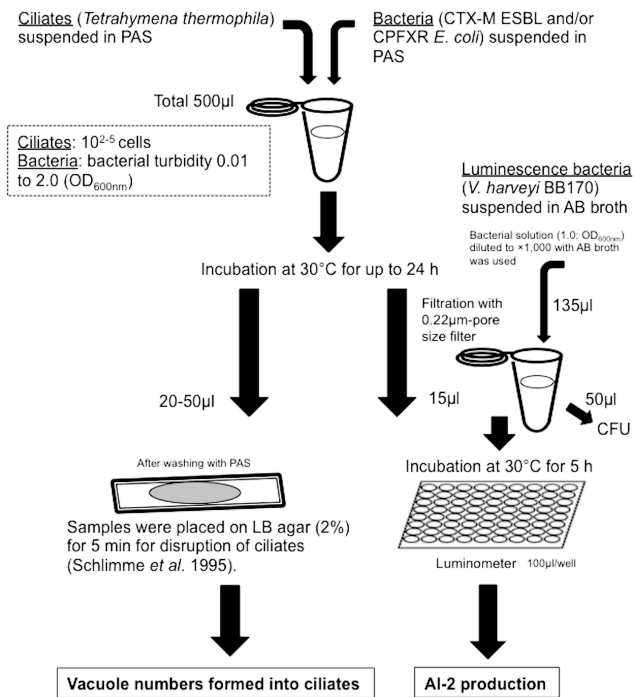


Fig. 1. Protocol with a mixed culture system for assessing vacuole numbers in ciliates and AI-2 production.

The mixed cultures were constructed as described previously (Matsuo et al., 2010). Assessment of ciliate vacuoles was performed microscopically, as previously described (Schlimme et al., 1995). CPFXR and/or CTX-M ESBL *E. coli* $\Delta luxS$ mutants were used in some experiments. The ratio of the bacteria in the mixed culture was 1:1.

extract at 37°C. The ciliates were also statically maintained in peptone-yeast extract-glucose broth containing 0.75% peptone, 0.75% yeast extract, and 1.5% glucose at 30°C, as described previously (Matsuo et al., 2010). The luminescent bacterium *Vibrio harveyi* BB170 was used for assessing AI-2 level in the culture. The luminescent bacteria were maintained in an Autoinducer Bioassay broth consisting of 3.7% brain-heart infusion, 1.25% NaCl, 1.23% MgSO₄ and 0.1 M L-arginine (Turovskiy and Chikindas, 2006). *E. coli* was also used for establishing *luxS* mutants (see below). Table 1 shows the bacteria used for this study.

Construction of *E. coli luxS* mutants. DNA fragments (~1 kbp) containing $\Delta luxS$ allele were obtained from CPFXR and CTX-M ESBL *E. coli* by polymerase chain reaction (PCR)-overlap extension, with two distinct primer sets for amplifying specific regions clipping the target sequence, *luxS* (Horton et al., 1989). The primer sets used for amplifying the $\Delta luxS$ allelic fragments were as follows: CPFXR *E. coli*, *EcluxS-F1/EcluxS-R1* and *EcluxS-F2/EcluxS-R2*; CTX-M ESBL *E. coli*, *EcluxS-F1/EcluxS-R2* and *EcluxS-F1/EcluxS-R3* (Supplementary Fig. S1, Table 2). The primer sets for the *E. coli* CPFXR strain were designed using a sequence for reference, the *luxS* locus of *E. coli* K-12 (accession number: U00096). Second, each of the in-frame *luxS* deletion fragments was generated from the $\Delta luxS$ allelic fragments by PCR with the following primer sets: CPFXR *E. coli*, *EcluxS-F1/EcluxS-R2*; CTX-M ESBL *E. coli*, and *EcluxS-F1/EcluxS-R3* (Fig. S1, Table 2). After digestion with *Bam*HI and *Sph*I, each of the

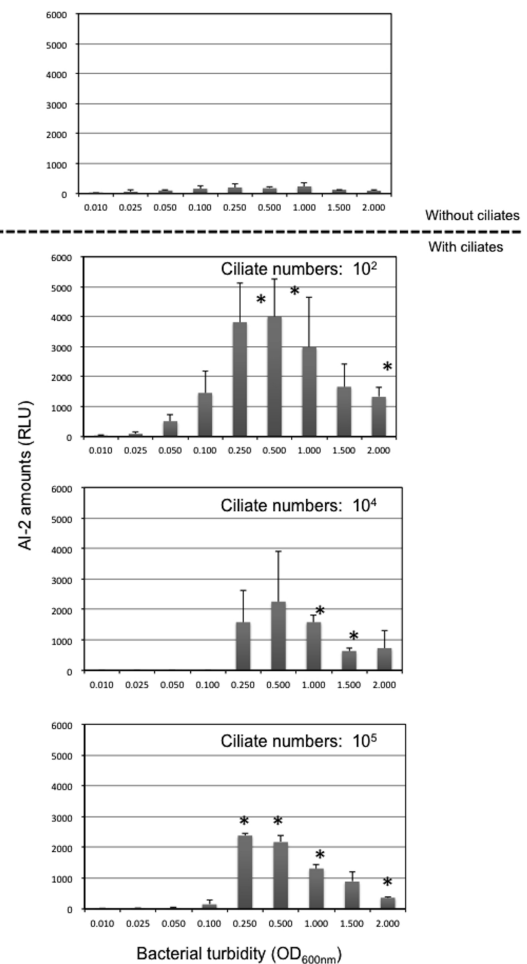


Fig. 2. Changes in AI-2 production in the mixed culture with or without ciliates.

Data show mean ±SD obtained from three experiments. **P* < 0.05 versus each of the values in mixed culture without ciliates.

fragments was cloned into pYAK1 plasmid vector (Horton et al., 1989) treated with *Bam*HI and *Sph*I [pYAK1c (CPFXR *E. coli*) and pYAK1e (CTX-M ESBL *E. coli*)]. We confirmed using direct sequencing that two distinct *luxS* allelic fragments were collectively identical to the target regions in the original sequences. After transformation, *E. coli* SM10 λ *pir* (Miller et al., 1988) carrying pYAK1c or pYAK1e (Kodama et al., 2002) was used for conjugation with CPFXR and CTX-M ESBL *E. coli*, respectively. The plasmid integrated trans-conjugants were selected on LB agar plates containing 40 µg/ml chloramphenicol, and the *luxS* mutants generated by second recombination were selected on LB agar containing 5% sucrose. Finally, the *luxS* deletions in the obtained clones were confirmed by PCR using a primer set, *EcluxS-CHF* and *EcluxS-CHR* (Fig. S2). Tables 1 and 2 show the plasmids and primer sets used for this study, respectively. The construction was confirmed using PCR with direct sequencing. Supplementary Fig. S3 summarizes the protocol for establishing *luxS* mutant.

Mixed culture of ciliates and bacteria. A mixed culture system for ciliates and bacteria was constructed as previously reported (Matsuo et al., 2010). As shown in Fig. 1, CTX-resistant ESBL *E. coli* and/or CPFXR-resistant *E. coli*

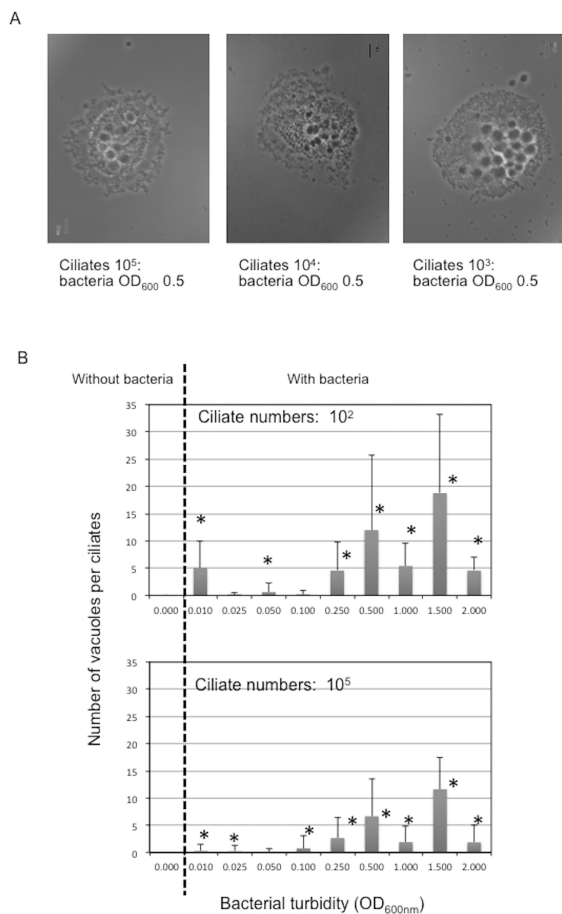


Fig. 3. Vacuole images and changes in vacuole numbers formed in ciliates.

A. Representative images showing vacuoles formed in ciliates (24 h post incubation). B. Changes in vacuole numbers formed in ciliates. Data show mean \pm SD obtained from three experiments. * $P < 0.05$ versus the values at each number of ciliates in the mixed cultures without bacteria (left column).

were incubated with or without *T. thermophila* IB into Page's amoeba saline (Na_2HPO_4 0.142 g, KH_2PO_4 0.136 g, NaCl 0.12 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.04 g, $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ 0.06 g, 1L) (Page, 1988) for up to 24 h at 30°C. Adequate amounts of suspension in the mixed culture with or without ciliates (total 500 μl) were collected for assessing vacuole numbers formed in ciliates (20–50 μl) and AI-2 production (15 μl). Each of the assessments was performed by the following protocols.

Assessment of AI-2 in the mixed culture. The amount of AI-2 in the mixed culture was estimated as follows. The solution was passed through a filter with 0.22- μm pore size. The filtered solution (15 μl) was mixed with a diluted solution of *V. harveyi* (135 μl). One hundred microliters of the solution was placed into the luminometer black plate and incubated for 5 h at 30°C. After incubation, the amount of luminescence produced in the solution was measured by a luminometer (Luminescencer-JNR II AB-2300; ATTO, Tokyo, Japan). The remaining solution was used for determining the number of *V. harveyi* organisms. The luminescence showing AI-2 level was expressed as relative light units (RLU) per bacterium (*V. harveyi*).

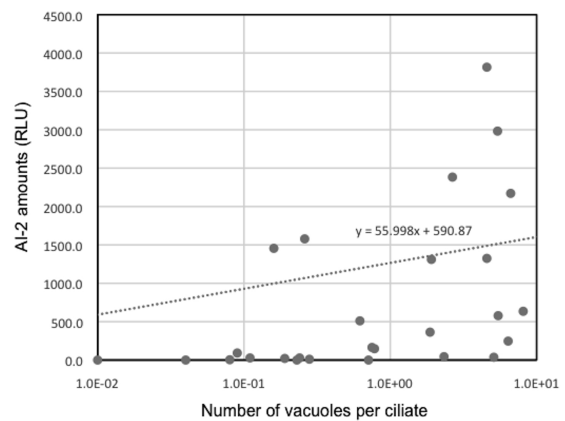


Fig. 4. Correlation between AI-2 level and number of vacuoles. Correlation index was 0.626, with a significant correlation ($p < 0.005$).

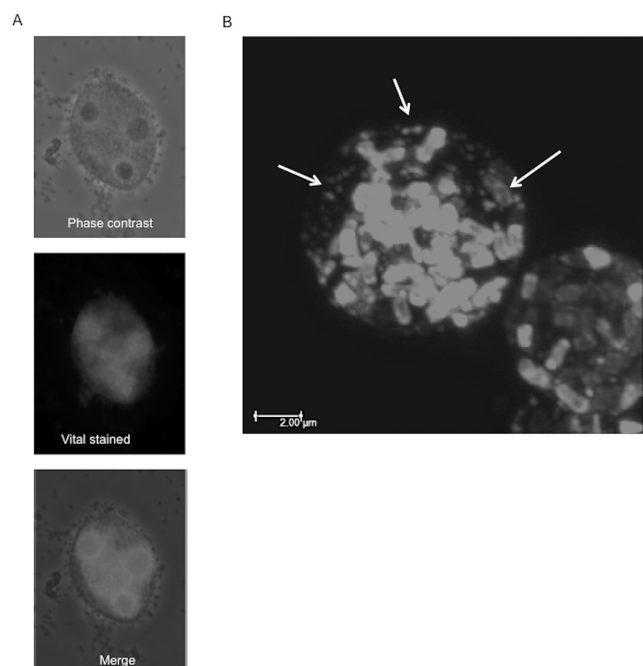


Fig. 5. Visualization of ciliate vacuoles in a mixed culture with vital-stained *E. coli*.

A. Representative images showing vital-stained *E. coli* packed in vacuoles of ciliates, visualized by conventional fluorescence microscopy (24 h post-incubation). B. Representative images showing vital-stained *E. coli* packed in vacuoles of ciliates, visualized by confocal fluorescence microscopy (24 h post-incubation). Arrows show disrupted bacterial fragments.

Assessment of vacuole numbers formed in ciliates. The number of vacuoles formed in ciliates was estimated as described previously (Schlimme et al., 1995). The mixed solution was gently placed on 2% LB agar solidified onto a glass slide, and incubated for 5 min at room temperature. After incubation, the vacuoles remaining on the glass slide were observed by light microscopy. The number of vacuoles per ciliate was estimated by observing more than 100 ciliates under light microscopy.

Assessment of bacterial localization in ciliate vacuoles. To confirm *E. coli* accumulation in ciliate vacuoles, ciliates and vital-stained CTX-M ESBL *E. coli* (fluorescence color:

Table 3. Changes of AI-2 production into mixed culture system of ciliates with *luxS* mutant.

Strain(s)	n*	AI-2 amount (fold change) [†]	p value [§]
With ciliates: 10 ²			
CTX-M ESBL <i>E. coli</i> -CPFX-R <i>E. coli</i>	3	4.96 ± 2.21] } < 0.05
CTX-M ESBL <i>E. coli</i> Δ <i>luxS</i> -CPFX-R <i>E. coli</i>	3	1.15 ± 1.15	
CTX-M ESBL <i>E. coli</i> -CPFX-R <i>E. coli</i> Δ <i>luxS</i>	3	3.46 ± 1.84	
CTX-M ESBL <i>E. coli</i> Δ <i>luxS</i> -CPFX-R <i>E. coli</i> Δ <i>luxS</i>	3	0.02 ± 0.04	

* Experimental times

[†] 24h/0h[§] All combinations unconnected with bold line are "not significant".

red) were used. After fixing bacteria with 4% paraformaldehyde, bacterial localization in ciliates was assessed using conventional (with or without deconvolution) or confocal fluorescence microscopy. Fluorescence vital staining for bacteria was performed with the PKH-26 labeling kit (MINI26; Sigma, St Louis, MO, USA) (Wallace et al., 2008).

RNA extraction and quantitative (q)RT-PCR. To assess expression of *luxS* (encoding AI-2) and *ydgG* (transporter for controlling bacterial export of AI-2), qRT-PCR was performed. Total RNA was extracted from mixed culture of ciliates and *E. coli* using High Pure RNA Isolation Kit (Roche, Indianapolis, IN, USA). Reverse transcription of total RNA was performed with ReverTraAce qPCR RT Master Mix (Toyobo, Osaka, Japan). Resultant cDNAs were amplified by SYBR Green Real-time PCR Master Mix (Toyobo) using the primer sets described in Table 2.

Statistical analysis. Experiments were repeated at least three times. Statistical analysis between values was performed with the unpaired Student's *t* test. A correlation index (*r*) with a regression curve was calculated by Spearman rank correlation. A value of *P* < 0.05 was considered statistically significant.

Results

Association of *E. coli* AI-2 accumulation in mixed culture with ciliates

Our previous study revealed that gene transfer between bacteria is induced by accumulation into vacuoles of ciliates (Matsuo et al., 2010; Oguri et al., 2011), possibly with an increase of AI-2, which is responsible for quorum sensing. We therefore assessed if AI-2 could be detected in the mixed culture system with vacuolar accumulation. AI-2 accumulation in mixed culture with ciliates was significantly increased when compared with that without ciliates (Fig. 2). While AI-2 appeared to decrease when the number of ciliates increased, the maximal AI-2 value was ~4,000 RLU per *V. harveyi* bacterium, and the fold change was 20 times more. Thus, ciliates are a stimulator for enhancing bacterial AI-2 accumulation in mixed culture supernatants, presumably evoking bacterial quorum sensing.

Association of *E. coli* AI-2 accumulation in mixed culture with ciliates with an increase in ciliate vacuole formation

We confirmed a possible association between vacuole

formation and engulfed bacterial AI-2 accumulation in ciliates. As shown in Fig. 3A, ciliate vacuoles were visualized, regardless of the number of ciliates. Multiplicity of infection (MOI) had no significant effect on the increase in vacuole formation in ciliates, which was consistent with the AI-2 accumulation data (Fig. 3B). The maximal vacuole number was ~20 vacuoles per ciliate at higher bacterial turbidity (OD₆₀₀ 0.5–2.0). We also estimated the correlation between vacuole formation in ciliates and bacterial AI-2 production, using the least squares method. There was a correlation between vacuole formation and AI-2 production (Fig. 4), with a correlation index *r* = 0.626 (*p* < 0.005). To confirm bacterial accumulation in ciliate vacuoles, we stained bacteria with PKH-26. The vital staining clearly revealed that the labeled bacteria had accumulated in the ciliate vacuoles (Fig. 5). Disrupted bacteria were also seen in the ciliate vacuoles, suggesting the leakage of AI-2 molecules from the bacteria into the culture medium, across the vacuolar membranes (Fig. 5B, arrows). We also found that the bacteria-filled vacuoles were frequently expelled into the culture supernatant (Fig. 6). More importantly, the expelled vacuoles appeared to be disrupted gradually (Fig. 6, arrows). These findings suggested that AI-2 accumulation in the mixed culture supernatants was associated with bacterial accumulation in the ciliate vacuoles, followed by expulsion of the vacuoles. However, we cannot deny that AI-2 accumulation may have been associated with induction of *luxS* and/or AI-2 transporter gene.

In addition, to deny a nonspecific phenomenon for AI-2 detection completely, we also confirmed if there was a difference in AI-2 accumulation between the wild-type and *luxS* mutant. The *luxS* mutants completely failed to accumulate AI-2 in the supernatants, as compared with wild-type *E. coli* (Table 3). The data clearly indicated that the bacterial AI-2 accumulation, but not nonspecific matter, occurred in a mixed culture with ciliates, which was possibly responsible for evoking peripheral bacterial quorum sensing.

E. coli AI-2 accumulation in mixed culture supernatant independently of *luxS* and *ydgG* gene expression

Since it is reasonable to predict upregulation of *E. coli luxS* gene encoding AI-2, depending on the presence of ciliates, we assessed if gene expression changed at two distinct time points after incubation in the mixed culture with or without ciliates. However, contrary to our expectation, no significant increase of *luxS* gene transcripts was

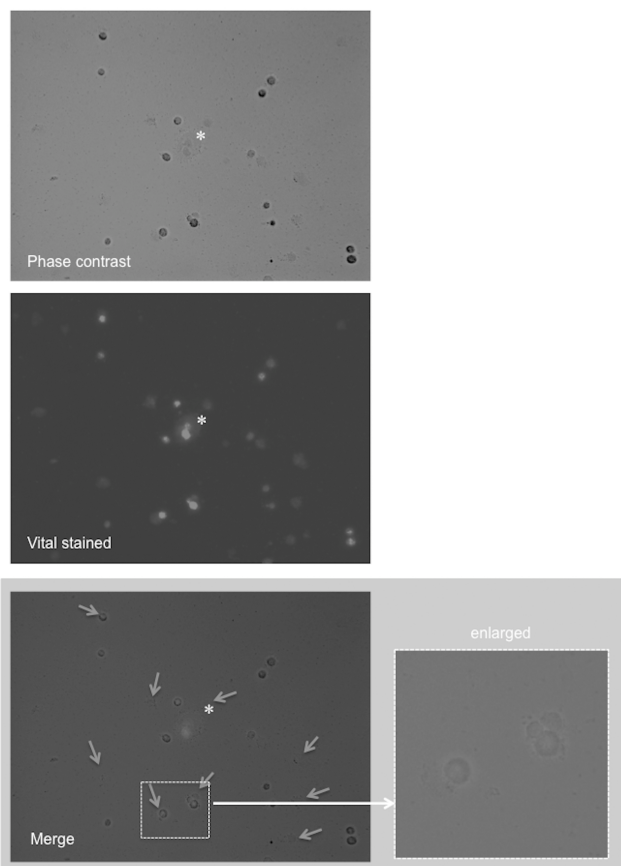


Fig. 6. Visualization of extracellular vacuoles showing vital-stained *E. coli* expelled by ciliates in a mixed culture.

Star indicates a ciliate. Arrows show disrupting vacuoles with leakage of bacteria. Representative images showing ciliates outside vacuoles, visualized by fluorescence microscopy equipped with deconvolution (Biozero, Keyence Japan, Osaka) (24 h post-incubation).

seen, regardless of the presence or absence of ciliates (Fig. 7A). Thus, the data indicated a minimal requirement of *luxS* gene expression for AI-2 accumulation in a mixed culture supernatant, although we cannot deny the possibility of upregulation of *luxS* gene at some earlier time point after incubation. Although the export mechanism of bacterial AI-2 produced by *luxS* remains unknown, several lines of evidence have revealed a possible way by which YdgG transporter could control AI-2 export in many bacteria, including *E. coli* (Herzberg et al., 2006; Pereira et al., 2012; Rettner and Saier, 2010). We next assessed if *ydgG* gene expression was associated with *E. coli* AI-2 accumulation in the mixed culture supernatant. No significant change in *ydgG* or *luxS* gene expression was observed (Fig. 7B). Thus, the data indicated that AI-2 accumulation in the mixed cultures was directly evoked by leakage of AI-2 from expelled vacuoles, following accumulation of disrupted bacteria, rather than an increased expression of *luxS* or *ydgG*.

Discussion

The mixed culture system of *E. coli* (clinical isolates: CTX-M ESBL and/or CPF-X-R *E. coli*) with *T. thermophila* was constructed, as described previously (Matsuo et al.,

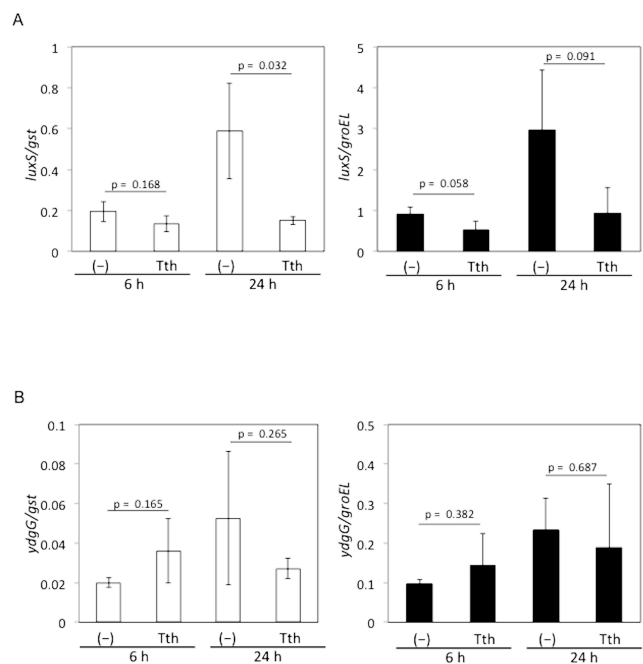


Fig. 7. Changes in *E. coli luxS* (A) and *ydgG* (B) gene expression in the culture with or without ciliates.

Expression of each gene was determined by qRT-PCR at 6 or 24 h post-incubation. Data express a ratio of each of the gene-expression values to either *gst* or *groEL* expression value. Tth, *T. thermophila*. Data shows means \pm SD obtained from at least three experiments. Statistical significance, $P < 0.05$ versus value of culture without ciliates (-).

2010). Because of a simple preparation, the bacterial suspensions were prepared according to the calibration curve determined by bacterial turbidity (OD₆₀₀). The ciliate vacuoles were microscopically visualized, as previously described (Schlimme et al., 1995); the vacuoles were distinct and clearly visible at 5 min after incubation in LB broth containing 2% agar at room temperature, permitting accurate vacuole counts. Furthermore, a luminescent bacterium, *V. harveyi* BB170, was the most popular biological tool for detecting AI-2 molecule, which is mainly responsible for quorum sensing depending on LuxS (Aharoni et al., 2008; Anand and Griffiths, 2003). Thus, these matters revealed that the AI-2 detection system used for this study was simple, with a high degree of accuracy, permitting certain reproducibility.

We found that the ciliates strongly promoted bacterial AI-2 accumulation in the mixed culture supernatant. Since there was a significant difference in AI-2 accumulation between the wild-type and *luxS* mutant, AI-2 detection in the mixed culture supernatant was not due to artificial error. Meanwhile, the total amount of AI-2 in the culture supernatant and vacuole numbers formed in the ciliates appeared to decrease as the number of ciliates increased. The data indicated that ciliate density rather than a ratio to bacteria (MOI) is important for effective AI-2 accumulation in the mixed culture supernatants, suggesting a requirement for effective bacterial prediction depending on ciliate density. Our hypothesis is supported by several examples of changes in bacterial prey rate depending on ciliate density (Bott, 1995; Clarholm et al., 2007; Suhr-Jessen and Orias, 1979).

As mentioned above, while AI-2 accumulation occurred into the mixed culture, expression of *luxS* and *ydgG* was unchanged, regardless of the presence or absence of ciliates. Vital staining revealed that the bacteria that accumulated in the ciliate vacuoles were disrupted. Vacuoles filled with bacteria were frequently expelled into the culture supernatant. Taken together with these findings, the AI-2 accumulation appeared to be induced by physiological factors such as bacterial disruption rather than biological stimulation. We therefore concluded that ciliates promoted bacterial AI-2 accumulation in the mixed culture supernatant via accumulation of disrupted bacteria in the ciliate vacuoles, independent of *luxS* or *ydgG* gene induction, followed by expulsion of the vacuoles into the culture supernatant. We now speculate about the mechanism of AI-2 accumulation in the mixed culture supernatants. First, engulfed bacteria are accumulated in ciliate vacuoles. Second, the bacteria in the vacuoles are partially digested into fragments, presumably resulting in leakage of AI-2 from bacteria into the vacuolar lumen. Third, some bacteria-containing vacuoles are expelled into the culture supernatant. Finally, hydrophobic AI-2 accumulated in the expelled vacuoles is easily translocated across the vacuolar plasma membrane into the culture supernatant.

Does bacterial AI-2 accumulation in the ciliate biotope have an impact upon survival of bacteria inhabiting the biotope? Although it remains an unanswered question, it is reasonable to assume that AI-2 accumulation provides some benefit to grazed bacteria surrounding ciliates. Several lines of evidence show that AI-2 accumulation is directly involved in the induction of bacterial virulence factors such as flagella (Verma and Miyashiro, 2013), type III (Lyczak et al., 2000) or IV (Leung et al., 2011) secretion systems, and biofilm formation (Solano et al., 2014). Furthermore, it is well known that AI-2 signaling regulates mixed bacteria rather than single species (Federle, 2009). Therefore, ciliate biotope may have a role of potential factory such as bacterial armaments to bacteria existing in the biotope, possibly connecting bacterial pathogenesis, although whether upregulation of bacterial virulence factors by AI-2 accumulation via ciliate predation occurs in actual environments remains unclear.

In conclusion, we found that ciliates promoted *E. coli* AI-2 accumulation in the ciliate biotope via accumulation of bacteria into ciliate vacuoles, followed by expulsion of the vacuoles, providing a novel relationship between ciliates and bacteria for quorum sensing. In natural environments, ciliate biotopes with bacterial AI-2 accumulation may provide a survival advantage to bacteria surrounding ciliates by evoking quorum sensing, which is also involved in bacterial pathogenesis.

Acknowledgments

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Supplementary Materials

Fig. S1. Primer design for the amplification and confirmation of *E. coli luxS* with framing regions.

Blue and red box surrounded by dashed lines show the primer sets for amplifying $\Delta luxS$ allelic fragments. Upper, CFPX-R *E. coli*. Lower, CTXM ESBL *E. coli*.

Fig. S2. Confirmation of the $\Delta luxS$ mutants by PCR amplification.

The images show two representative distinct mutants. M, molecular maker; WT, wild-type.

Fig. S3. Outline for construction of *E. coli luxS* mutants.

Supplementary figures are available in our J-STAGE site (<http://www.jstage.jst.go.jp/browse/jgam>).

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