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Lactobacillus acidophilus reduces expression of enterohemorrhagic *Escherichia coli* O157:H7 virulence factors by inhibiting autoinducer-2-like activity

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Abstract

Recent investigations have shown that many pathogenic bacteria have quorum sensing (QS) systems to coordinate virulence factors, pointing out a new, promising target for anti-microbial drugs. In the present study, by interfering with autoinducer (AI)-2-like activity, we investigated the effects of cell extracts isolated from *Lactobacillus* on the virulence of enterohemorrhagic *Escherichia coli* (EHEC) O157:H7, including attachment, biofilm formation, and the killing of a surrogate host, the nematode *Caenorhabditis elegans*. In the AI-2 bioassay, profound inhibitions of AI-2-like activity were exerted on EHEC O157:H7 ATCC 43894 in the presence of the 1.0% (w/v) *Lactobacillus acidophilus* A4 cell extract, whereas the growth of EHEC was not changed. By adding the cell extract, inhibitory effects were exhibited on epithelial cell attachment, as well as on biofilm formation on abiotic surfaces, whereas significant reductions in EHEC O157:H7 pathogenicity were not observed in the *C. elegans* nematode *in vivo* model. In the comparative proteomic analysis, the 1.0% cell extract down-regulated expression of several virulence factors controlled by AI-2-like activity. In particular, these proteins have consistent roles in membrane associated functions and sulfur metabolism.

Keywords: Autoinducer-2-like activity; EHEC O157:H7; *Lactobacillus acidophilus*; Attachment; Biofilm formation; Two-dimensional gel electrophoresis

1. Introduction

Bacteria use quorum sensing to regulate certain forms of gene expression by sensing their population density via small signaling compounds called autoinducers (AI) that are excreted into the environment (Xavier & Bassler, 2005). Due to its common role in quorum sensing, AI-2 signals have recently been the subject of much research. The AI-2 compound exists in many different species. For example, 35 of the 89 fully sequenced bacterial strains have the *luxS* gene (Xavier & Bassler, 2003); however, its roles within bacteria are not clearly understood. Recent studies have suggested AI-2 regulates virulence factors (Sperandio, Mellies, Nguyen, Shin, & Kaper, 1999), the ATP binding cassette (ABC) transport system (Taga, Semmelhack, & Bassler, 2001), motility (Sperandio, Torres, Giron, & Kaper, 2001), and biofilm formation (McNab et al., 2003). Therefore, due to their essential role in the expression of multiple virulence traits in various pathogens, including EHEC O157:H7, QS systems are clearly potential antibacterial drug targets (Rasmussen et al., 2005). Recently, in the best-studied example of AI-2 inhibition, the brominated furanones isolated from a marine alga, *Delisea pulchra*, inhibited several multicellular behaviors of Gram-negative bacteria such as swarming (Gram et al., 1996; Ren, Sims, & Wood, 2001), bioluminescence (Manefield, Harris, Rice, de Nys, & Kjelleberg, 2000), and biofilm formation (Hentzer et al., 2002;

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Ren, Bedzyk, Ye, Thomas, & Wood, 2004) without affecting general growth. It was also demonstrated that furanones inhibit cell to cell communication systems based on AI-1 (Ren et al., 2001). However, these furanone compounds consist of halogens that give them limitations for human use. Therefore, searches for natural products, as food-grade materials, that may be able to efficiently inhibit AI-2 signaling (Choo, Rukayadi, & Hwang, 2006) are required.

Probiotic bacteria including *Lactobacillus acidophilus* are live microorganisms belonging to the normal flora with no pathogenicity, but with functions of importance to the health and well being of the host cells. It is extensively accepted that these bacteria might control or prevent infections of pathogens (Kim et al., 2006b; Oh, Kim, & Worobo, 2000).

In the present study, we determined the effects of *L. acidophilus* A4 cell extract on EHEC O157:H7 pathogenesis in relation to motility, attachment, biofilm formation, and killing of the nematode *Caenorhabditis elegans*. In addition, proteomic analysis by two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) systems was performed to compare, at the protein level, the expression profiles of both the wild-type strain EHEC O157:H7 43894 and its *luxS* deficient mutant, as well as to compare the gene expression profiles with and without the extracts of *L. acidophilus*.

2. Materials and methods

2.1. Bacterial strains, plasmid, and culture media

The bacterial strains used in this study were obtained from the Dairy Food Microbiology Laboratory at Korea University (Seoul, Korea; Table 1). The parent EHEC O157:H7 ATCC 43894 strain and *luxS*-deficient strain (the *luxS* gene was inactivated *in vitro* by deletion of about 500-bp internal region using a two PCR fragments containing regions of *luxS* upstream and down stream, followed by insertion of the kanamycin cassette from pUC4K; personal communication, Park et al., 2006) were routinely cultured at 37 °C in Luria-Bertani broth (Difco, Sparks, MD, USA). Attachment experiments were performed using strains harboring the pKEN-gfpmut2 plasmid (Kim et al., 2007) to visualize the attachment phenotypes in HT-29 epithelial cells. When appropriate, kanamycin and ampicillin were added to the growth medium at concentrations of 50 μg/ml and 100 μg/ml, respectively. *Vibrio harveyi* BB152 and BB170 strains were used to identify AI-2-like activity and were grown aerobically at 30 °C in autoinducer bioassay (AB) medium (Surette & Bassler, 1998). *L. acidophilus* A4 (isolated from swine; Kim et al., 2006b) was cultured in de Man, Rogosa Sharpe (MRS) broth (Difco, Detroit, MI, USA) at 37 °C for 18 h.

Table 1
Bacterial strains and plasmid used in this study2

Strains and plasmid	Genotype and description	Reference
Strains		
43894	EHEC O157:H7 (stx[a]-1 and stx-2)	ATCC[b] Kim et al. (2007)
43894-*luxS*	*luxS* deficient strain of 43894	Park et al. (unpublished)
43894-GFP	43894 with plasmid pKEN-gfpmut2	Kim et al. (2007)
43894 *luxS*-GFP	43894-*luxS* with plasmid pKEN-gfpmut2	This study
BB152	*V. harveyi luxMN* (AI-1$^-$, AI-2$^+$)	Surette and Bassler (1998)
BB170	*V. harveyi luxN::Tn5* (sensor-1$^-$, sensor-2$^+$)	Surette and Bassler (1998)
OP50	*E. coli*, Laboratory food source for *C. elegans*	Tenor et al. (2004)
A4	*Lactobacillus acidophilus* strain	Kim et al. (2006b)
Plasmid		
pKEN-gfpmut2	Am^r, expression of enhanced GFP protein	Cormack, Valdivia, and Falkow (1996)

[a] stx, shiga-like toxin.
[b] ATCC, American Type Culture Collection.

2.2. Preparation of cell-free supernatant and detection of AI-2 activity

The conditioned medium (CM) were prepared by methods described previously (Kim et al., 2007). At 6 h incubation point, EHEC O157:H7 ATCC 43894 cultures were taken and centrifuged (14,000*g*, 5 min, 4 °C) and the supernatant was further clarified by filtration (0.22 μm; Sartorious, Germany). The presence of AI-2-like activity in the CM was tested using the *V. harveyi* BB170 reporter strain (AI-1 sensor negative and AI-2 sensor positive) as method described previously (Kim et al., 2007). The CM derived from *V. harveyi* BB152 (expresses AI-2, but not AI-1) and the *luxS* deficient strain served as the positive and negative controls, respectively, to investigate the effect of AI-2-like activity. Briefly, 10 μl of CM in the presence or absence of active components isolated from *L. acidophilus* A4 was added to 96-well microtiter dishes. The *V. harveyi* reporter strain BB170 diluted 1:5000 into fresh AB medium, and 90 μl of the diluted cells was added to the wells containing the prepared CM samples. The microtiter dishes were shaken in a rotary shaker at 175 rpm at 30 °C. The time course of bioluminescence was evaluated using a luminometer (Wallac model 1420 multilabel counter; Perkin Elmer, Boston, MA, USA). The AI-2 activity was normalized as 100% activity by the light production of *V. harveyi* BB152 as positive control.

2.3. Preparation of active components from L. acidophilus strains

The cell extract of *L. acidophilus* A4 were prepared with method as described previously (Choo et al., 2006). Briefly, overnight *L. acidophilus* A4 cultures were harvested by centrifugation (3500*g*, 30 min, 4 °C) and washed twice with phosphate-buffered saline (PBS). The cell pellet was

washed and resuspended in PBS prior to 20 min (at 1 min intervals) of ultrasonic disruption in a cooled water bath (4 °C), then supernatants (cell extract fractions) were subsequently recovered by centrifugation (14,000*g*, 1 h). In addition, two types of exopolysaccharides (EPS; cell-bound EPS and released EPS) from *L. acidophilus* A4 were prepared by previously described methods (Kim et al., 2006a).

All samples were adjusted to 1.0% (w/v) concentrations with medium or conditioned medium, sterilized by filtration (0.22 μm pore size; Sartorious, Gottingen, Germany), and stored at −70 °C until further experiments. Growth curves in the absence or presence of active components were monitored spectrophotometrically by a Shimazu spectrophotometer (UV-1600; Shimadzu, Tokyo, Japan) by measurement of optical density at 600 nm at same set time points.

2.4. Motility and attachment assay

The freshly grown EHEC bacterial colonies were transferred with a sterile toothpick into swim agar (1% tryptone, 0.5% NaCl, 0.25% agar) or swarm agar (1% tryptone, 0.5% NaCl, 0.5% agar). When necessary, the 1.0% cell extract from *L. acidohilus* was added to the motility medium. The plates were incubated face up for 12 and 24 h at 30 °C, and motility was then assessed qualitatively by examining the circular turbid zone formed by the bacterial cells migrating away from the point of inoculation.

The human intestinal epithelial cell line HT-29, have been used extensively in the study of human enterocytic function, was obtained from the Korea Cell Line Bank (KCLB, Seoul, Korea). The advantages of this *in vitro* model are that they express morphological and functional differentiation and show characteristics of mature enterocytes, including polarization and functional brush border (Coconnier, Klaenhammer, Kerneis, Bernet, & Servin, 1998). The cells were routinely cultured in RPMI 1640 medium (Gibco BRL, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Prior to the attachment assay, the HT-29 monolayers were washed three times with PBS to remove culture medium and non-attached cells. The prepared *luxS* mutant and EHEC 43894 strains (10^6 CFU/ml), with or without 1.0% cell extract, were added at various times at 37 °C in an atmosphere of 5% CO_2. After attaching for 3 and 5 h, the monolayers were washed six times with PBS to remove the non-attached bacteria. The attached cells were lysed using 0.1% Triton X-100 for 5 min. Serial dilutions of the mixture were plated onto MacConkey agar (Difco, USA) and incubated at 37 °C for 48 h. The attachment ability was determined by counting CFUs per ml. In analogous experiments, fluorescent images were directly determined with fluorescence microscopy (Olympus IX71, Japan), at the same incubation times, to observe the changes in attachment phenotype by AI-2-like activity and the cell extract.

2.5. Biofilm formation assay

The biofilm formation protocol was adapted from Kim et al. (2006c). Overnight cultures of the EHEC wild-type and *luxS* mutant were washed twice with PBS and inoculated at 10^6 CFU/ml in fresh 1/2 diluted LB that was supplemented with the 1.0% (w/v) cell extract. After incubation at 30 °C for 48 h without agitation, the microplates (polystyrene and polyvinyl chloride) were thoroughly rinsed twice with sterile distilled water, and a 0.1% (w/v) solution of crystal violet (CV) was added to stain the attached cells. Following staining at room temperature for 20 min, the CV was removed and the wells were rinsed with sterile water three times, and the dye was solubilized with 95% ethanol. The absorbance of the solubilized dye was subsequently determined at 595 nm.

2.6. Nematode C. elegans killing assay

The *C. elegans* killing assay was performed as initially described by Tenor, McCormick, Ausubel, and Aballay (2004) with some modification. Briefly, EHEC O157:H7 43894 was grown in 5 ml of LB liquid culture in the absence or presence of 1.0% (w/v) cell extract overnight with shaking. The culture samples (15 μl each) were then spread on Nematode growth medium (NGM) agar plates (Brenner, 1974; diameter of each, 30 mm) containing the 1.0% (w/v) cell extract. In the present study, instead of wild-type Bristol N2 worms, we tested the killing of *C. elegans* glp-4 mutant worms. *C. elegans* glp-4 animals have normal morphology and brood sizes at 15 °C, but do not make gonads and are unable to produce eggs at 25 °C (Mylonakis, Ausubel, Perfect, Heitman, & Calderwood, 2002). After cultivated at 15 °C, the glp-4 young adult worms (12 each) were finally transferred to plates, which were then sealed with parafilm and incubated at 25 °C. The number of living worms per plate was determined at various time points for six days with light microscopy (Olympus CH30, Tokyo, Japan). The nematodes were considered dead when they failed to respond to tapping of the plate. Each experimental condition was tested in duplicate or triplicate. *E. coli* OP50 was used as the control (Kim et al., 2007; Tenor et al., 2004).

2.7. Proteomic analysis

The EHEC O157:H7 43894 strain and *luxS* mutant were grown in 250 ml of LB medium, with or without 1.0% (w/v) cell extract, for 6 h at 30 °C. The protein preparation and two-dimensional gel electrophoresis (2-DE) procedures were performed with the same methods described by Kim et al. (2007). The stained gels were scanned using a densitometry instrument (800 × 1600 dpi, UMAX, UTA 2100XL, USA) and the spots analyzed by PDQuest software (Bio-Rad) according to manufacturer's instructions. Three gels resulting from 2-DE experiments were obtained and two gels of good quality were used for analysis. Using

computer-aid analysis, only significant spot intensity changes (at least 2.0-fold) were considered and selected using for further identification. The protein spots were enzymatically digested in-gel using modified porcine trypsin (Promega, USA). For protein identification, MALDI-TOF/MS was employed using the method described by Kim et al. (2007). In the case of some protein spots (YnhG), protein information was obtained by modifying the digest supernatant using a chemically assisted fragmentation (CAF)-MALDI sequencing kit (Amersham Biosciences, Uppsala, Sweden). The fragment masses obtained from CAF-MALDI could then be submitted to Sonar in the Ettan MALDI-TOF software, or a similar protein identification search engine (PepFrag; http://prowl.rockefeller.edu/). The mass of the native (non-derivatized) peptide and five fragment masses, or more (depending on the protein), are needed for protein identification. By submitting the amino acid sequence, the protein can be identified by a homology search using, for example, ProteinInfo™ (www.proteometrics.com), or in a BLAST search using the ExPASy Molecular Biology Server (www.expasy.ch).

2.8. Statistical analysis

All experiments were replicated at least three times; otherwise, the number of repeated times was stated in the figure legend. Statistical significance was assessed by ANOVA, followed by Duncan's test with the SAS software package (Ver. 9.1). The level of significance was accepted at $p < 80.05$.

3. Results and discussion

3.1. Production of AI-2-like activity by EHEC O157:H7 strains

The wild type (WT) of EHEC O157:H7 ATCC 43894 (hereafter referred to as EHEC 43894), in the presence or absence of the 1.0% (w/v) *L. acidophilus* A4 cell extract, and the *luxS* deletion mutant, had similar growth rates (data not shown). Consistent with previous reports (Kim et al., 2007; Surette & Bassler, 1998), the WT expressed maximal levels of AI-2-like activity at 6 h of incubation, approximately mid-exponential phase, and the activity decreased with further growth. In contrast, the *luxS* mutant strain had dramatically reduced AI-2-like activity compared to the WT strain, and at 6 h, the mutant's activity was only 5.2% that of the parent strain (Fig. 1A). Similar reports were observed on the *luxS* mutant of other pathogenic bacteria, including *Listeria monocytogenes* (Sela, Frank, Belausov, & Pinto, 2006), *Streptococcus mutans* (Merritt, Qi, Goodman, Anderson, & Shi, 2003), and *Helicobacter pylori* (Osaki et al., 2006).

Interestingly, profound inhibitions of AI-2-like activity were exerted on EHEC 43894 in the presence of the 1.0% (w/v) cell extract (approximately 20% that of the parent strain). However, there was no significant inhibition in the other treatments such as the cell-free supernatants and the cell-bound and released EPS at the same concentrations. In addition, attenuated growth of *V. harveyi* BB170 was observed in the presence of the 10.0% cell extract (data not shown). Finally, the 1.0% cell extract concentration was used as the AI-2-like activity inhibitor in further experiments. In addition, we checked whether adding 1.0% cell extract in EHEC 43894 could interfere with bacterial growth. As expected, there were no significant differences in the growth curves of EHEC 43894 in the absence or presence of the 1.0% cell extract at all incubation times (Fig. 1B), which suggests that the existence of the cell extract in the culture medium did not affect the growth of EHEC 43894.

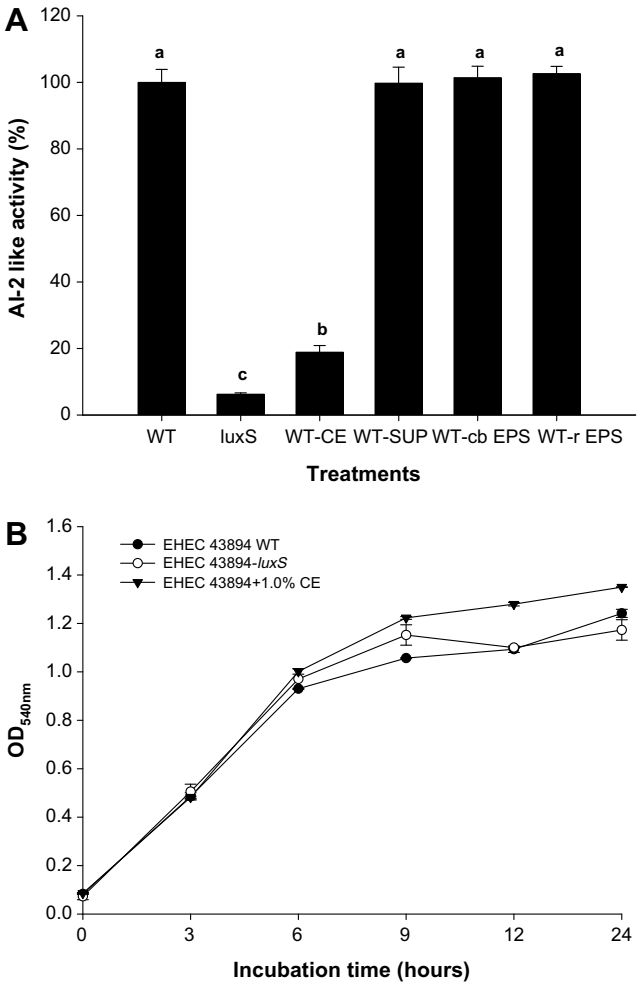

Fig. 1. (A) Growth curves of the *luxS* deficient and EHEC 43894 wild-type strains in the absence or presence of 1.0% cell extract isolated from *Lactobacillus acidophilus* A4. (B) Effects of cell extract on the induction of AI-2-like activity. Reporter strains *V. harveyi* BB170 were grown in AB medium overnight, diluted 1:5000 into the fresh AB medium. Then, the conditioned medium (CM) from the *luxS* mutant and EHEC 43894 cultures, in the presence or absence of the indicated materials (CE, cell extracts; SUP, cell-free supernatants (pH 6.5); cb-EPS, cell bound EPS; and r-EPS, released EPS), was added at a concentration of 1.0% (w/v). The bioluminescence was measured 4 h later with a luminometer (Wallac model 1420 multilabel counter).

Prior to now, there have been few reports on natural compounds that inhibit AI-2, except (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone (furanone) from the marine alga *D. pulchra* (Ren et al., 2001). To our knowledge, this is the first reported investigation of AI-2-like activity inhibition by the cell extract isolated from a *Lactobacillus* strain.

3.2. Effects of AI-2-like activity and cell extract on the virulence of EHEC O157:H7

Motility and attachment ability are important means for pathogens to exert their virulence (Sperandio et al., 1999). However, in the treatments with the *luxS* mutant and EHEC, in the presence or absence of the 1.0% (w/v) cell extract, motility ability was not significantly changed (data not shown). Similar to a previous report (Ren et al., 2004), these results indicate that AI-2-like activity is not entirely necessary for motility reaction in EHEC O157:H7, although it has been found to alter the regulation of several motility-associated genes.

In this work, attachment experiments were performed with human epithelial HT-29 cells as an intestinal model, in order to determine if the cell extract could reduce the attaching ability of EHEC O157:H7 by the inhibition of AI-2-like activity. Under the absence of AI-2-like activity (*luxS* mutant), the attachment ability of EHEC O157:H7, an important prerequisite for virulence manifestations, decreased on the HT-29 cells at 5 h of incubation compared to WT; whereas some increased attachment was shown at 3 h of incubation. Also, the 1.0% (w/v) cell extract decreased the attachment of EHEC O157:H7 by 35% on HT-29 cells at 3 h of incubation, but not at 5 h (Fig. 2A). Additionally, EHEC O157:H7 strains expressing GFP were used to visualize alterations of the attaching phenotype. The EHEC O157:H7 WT formed attachment aggregates

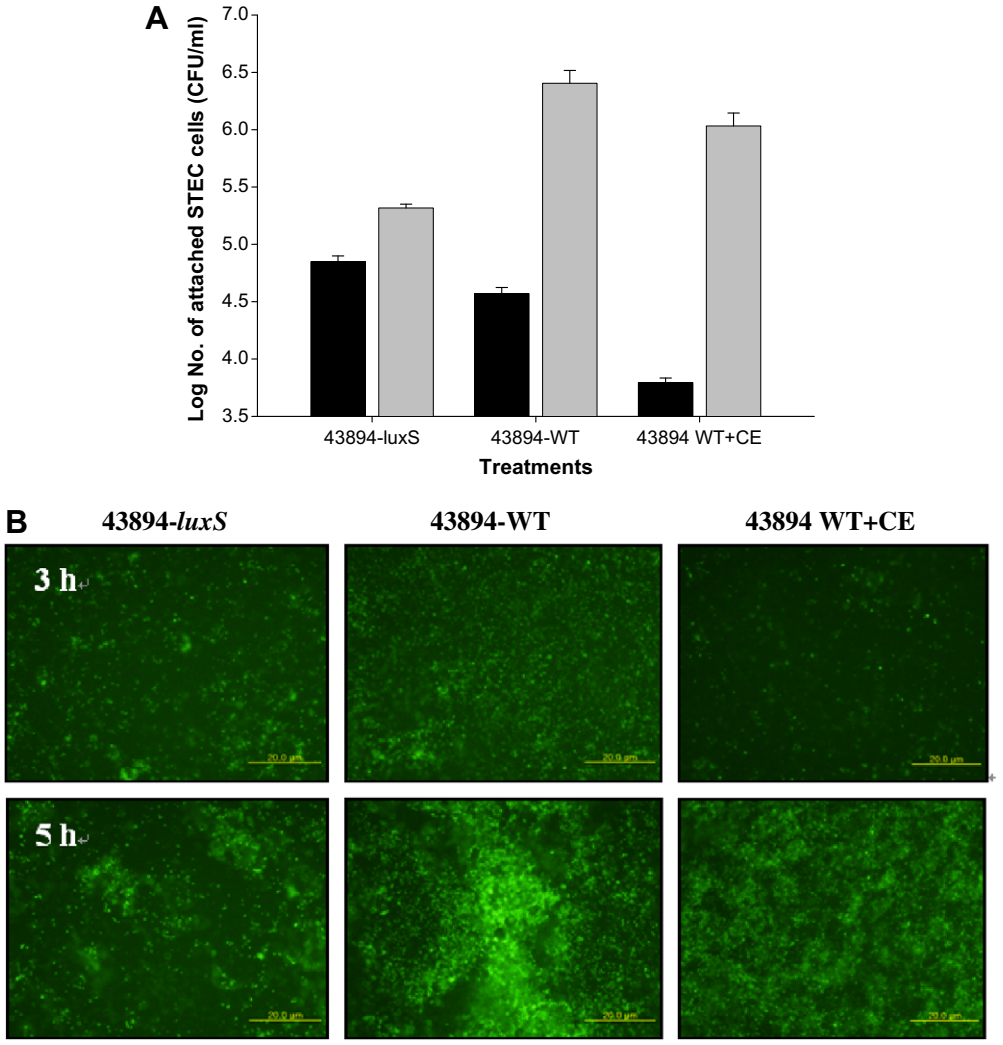

Fig. 2. Effects of cell extracts isolated from *Lactobacillus acidophilus* A4 on the attachment of EHEC O157:H7 43894 to HT-29 epithelial cells (A, black bars: 3 h and grey bars: 5 h). Fluorescence images of EHEC 43894-*luxS* and EHEC 43894 in the presence or absence of 1.0% (w/v) cell extract on HT-29 cells at the same incubation times (B). Following the incubation of each cell with the HT-29 cells, the detachment was carried out according to the experimental procedure in Section 2. Mean values for three independent experiments are shown with standard deviations (SD).

more rapidly (Fig. 2B) compared to the *luxS* mutant. Interestingly, attachment was inhibited by the presence of the 1.0% (w/v) cell extract with significant decreases in the attachment aggregates. These findings indicate that factors related to attachment and their development procedures are regulated by AI-2 signaling. Until now, few studies have reported on the relationship between AI-2-like activity and attachment. Kanamaru, Kanamaru, Tatsuno, Tobe, and Sasakawa (2000) reported that the EHEC O157:H7 Sakai strain, which overexpresses SdiA, a QS-negative regulation factor, had markedly impaired ability to form microcolonies on Caco-2 cells. When linked to the previous investigation (Kim et al., 2007; Sperandio et al., 2001), AI-2-like activity encoding by the *luxS* gene seems to play an important role in the attachment of EHEC O157:H7. Consequently, in the present study, it appears that cell extracts isolated from *L. acidophilus* A4 may inhibit the initial attachment of EHEC O157:H7 to intestinal epithelial cells, by partially affecting AI-2-like activity.

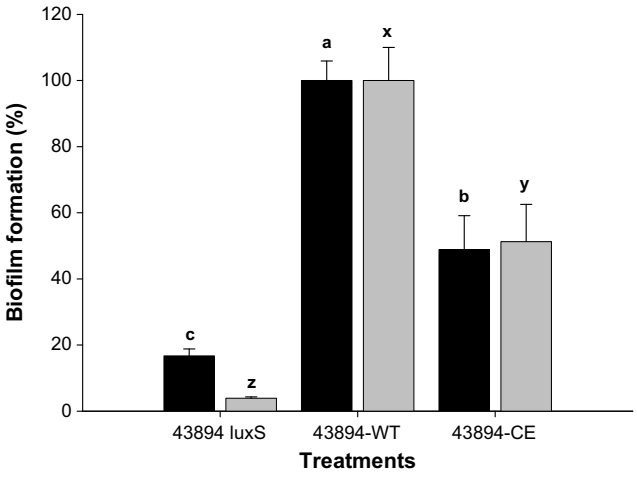

Fig. 3. Effects of cell extracts isolated from *Lactobacillus acidophilus* A4 on biofilm formation. Quantification of biofilm formation was carried out according to the experimental procedure in Section 2 on abiotic surfaces under static aerobic growth conditions in 1/2 diluted LB medium for 48 h. The polystyrene (black bars) and polyvinyl chloride (PVC) (grey bars) were used as abiotic surfaces. Mean values for three independent experiments are shown with standard deviations (SD).

3.3. Effect of AI-2-like activity and cell extracts on the biofilm formation of EHEC O157:H7

In general, it was previously shown that AI-2-like activity, a cross-species quorum sensing signal, stimulated biofilm formation in *E. coli* hosts (Gonzalez Barrios et al., 2006). However, until now, the apparent role of AI-2-like activity was not understood. Interestingly, a recent report indicated that the *luxS* mutant has no effect on biofilm formation (Daines et al., 2005). Furthermore, several reports demonstrated that biofilm formation was induced more in the QS mutant strain of pathogenic bacteria than the wild-type counterpart (Sela et al., 2006). In this study, since the addition of *L. acidophilus* A4 cell extracts significantly reduced AI-2-like activity and attachment ability in EHEC 43894, we tested whether the biofilm formation of EHEC 43894 was also influenced.

To investigate whether AI-2-like activity caused alterations in EHEC O157:H7 biofilm formation, we first measured the biofilm formation abilities of the WT strain and *luxS* mutant on abiotic surfaces. As expected, the *luxS* mutant made 20% less biofilm than the isogenic WT strain, thus indicating that AI-2-like activity affects biofilm formation (Fig. 3). We also tested whether the *L. acidophilus* A4 cell extract exhibited an inhibitory effect on regular biofilm formation. EHEC O157:H7 biofilms were allowed to form for 48 h on both polystyrene and PVC surfaces, in which the medium contained either no cell extract or 1.0% (w/v) cell extract. In Fig. 3, compared to the WT strain, one can see that biofilm formation was significantly repressed, approximately 50%, by the addition of the 1.0% (w/v) cell extract on both the polystyrene and PVC surfaces. Previously, some research groups showed that furanone inhibited genes related to AI-2 quorum sensing (Ren et al., 2004), and that it inhibited biofilm formation of *E. coli* (Ren et al., 2001). However, in contrast to furanone, they also demonstrated that ursolic acid (from *Diospyros dendo*, the tree used for ebony from Gabon, Africa) at a concentration of 10 μg/ml, in 96-well plates and a flow chamber system, inhibited biofilms without interfering with quorum sensing (Ren et al., 2006). Therefore, as similar to the effects of furanone, the cell extract of *L. acidophilus* A4 indirectly attenuated biofilm formation of EHEC O157:H7, at least in part, by interfering with AI-2 like activity.

3.4. Effect of AI-2-like activity and cell extracts on the survival of C. elegans

Previous data (Rasmussen et al., 2005) have shown that in 5 h of incubation time, the QS-deficient mutant of *Pseudomonas aeruginosa* killed fewer numbers of nematodes compared to its parent strain. These data provide some evidence, at least in part, that the stimulation of *C. elegans* killing in the presence of AI-2-like activity may be caused by the induction of virulence factors. However, during six days of incubation, no profound induction of life span was observed in the killing-assay of *C. elegans* with the *luxS* mutant compared to the WT strain in our present work. Also, at the same incubation time, when the medium was supplemented with the 1.0% (w/v) cell extract of *L. acidophilus* A4, there was no significant difference on worm survival (data not shown). Interestingly, it was recently reported that the paralysis and killing of *C. elegans* by pathogenic *E. coli* did not require direct contact, suggesting that a secreted toxin mediated the effect (Anyanful et al., 2005). At this time, ongoing studies are evaluating enhanced survival of *C. elegans in vivo* models in the presence of cell extracts, using a stx-deficient mutant strain.

3.5. Proteome analysis

Using 2-DE coupled with MS techniques, we investigated the inhibition of AI-2-like activity by the *L. acidophilus* A4 cell extract at the functional protein level, and the findings were then compared with our earlier study (Kim et al., 2007).

To date, there were few reports on the cellular response of quorum sensing signals associated EHEC strain at the level of protein or mRNA. On the other hand, regardless of non-EHEC strain, DeLisa, Wu, Wang, Valdes, and Bentley (2001) examined on the global changes in mRNA abundance elicited by the AI-2 signaling molecule through the use of a *luxS* deficient mutant that was unable to synthesize AI-2 compared to parent *E. coli* W3110 strain. Interestingly, they observed that 242 genes, comprising approximately 5.6% of the *E. coli* genome populations, exhibited significant transcriptional changes (either induction or repression) in the presence of AI-2.

However, in our proteome works, only a few protein spots were significantly induced or repressed by *luxS* mutant or wild-type in the presence of cell extracts of 1.0% cell extract of *L. acidophilus* A4 compared to wild-type EHEC strain. Among the approximately 550 spots detected, only twelve spots were differentially up (four spots) or down (eight spots) regulated under both *luxS* deficient mutant and wt in the presence of cell extract using computer-aid analysis (data not shown). In our previous study (Kim et al., 2007), we reported that total twenty-two protein spots with 2.5-fold increase or decrease in amounts were detected from EHEC O157:H7 43894 in the presence of AI-2 like activity compared to the control (eighteen and four spots were up- and down-regulated, respectively). As a comparison of this previous report, we showed that the specific five protein spots (NifU, DsbA, FlgI, MdaB, and DsbA), which were significantly up-regulated in the presence of AI-2 like activity, were observed on the down-regulation under both *luxS* deficient mutant and WT in the presence of cell extract in this study.

Four protein spots (NifU, DsbA, FlgI, and MdaB) that were induced by crude AI-2-like activity in our earlier study were down-regulated in the *luxS* mutant. The finding of these genes supports the possible role of AI-2-like activity in EHEC pathogenesis. Interestingly, in the presence of the 1.0% (w/v) cell extract of *L. acidophilus* A4, these proteins were reduced compared to the parent strain (Fig. 4). Among these, it was reported that *flgI*, controlled by AI-2-like activity, was significantly repressed in the presence of furanone in a DNA microarray (Ren et al., 2004). Therefore, our results indicate that several surface-associated proteins identified as part of the AI-2-like activity regulon, are repressed by the cell extract. Moreover, in addition to the cell extract, which reduced expression of these proteins, there is an alternative approach for controlling bacterial infections associated with AI-2-like activity.

DsbA is known to be required for catalyzing the oxidative folding and assembly of many virulence factors (Tan, Mahajan-Miklos, & Ausubel, 1999). Interestingly,

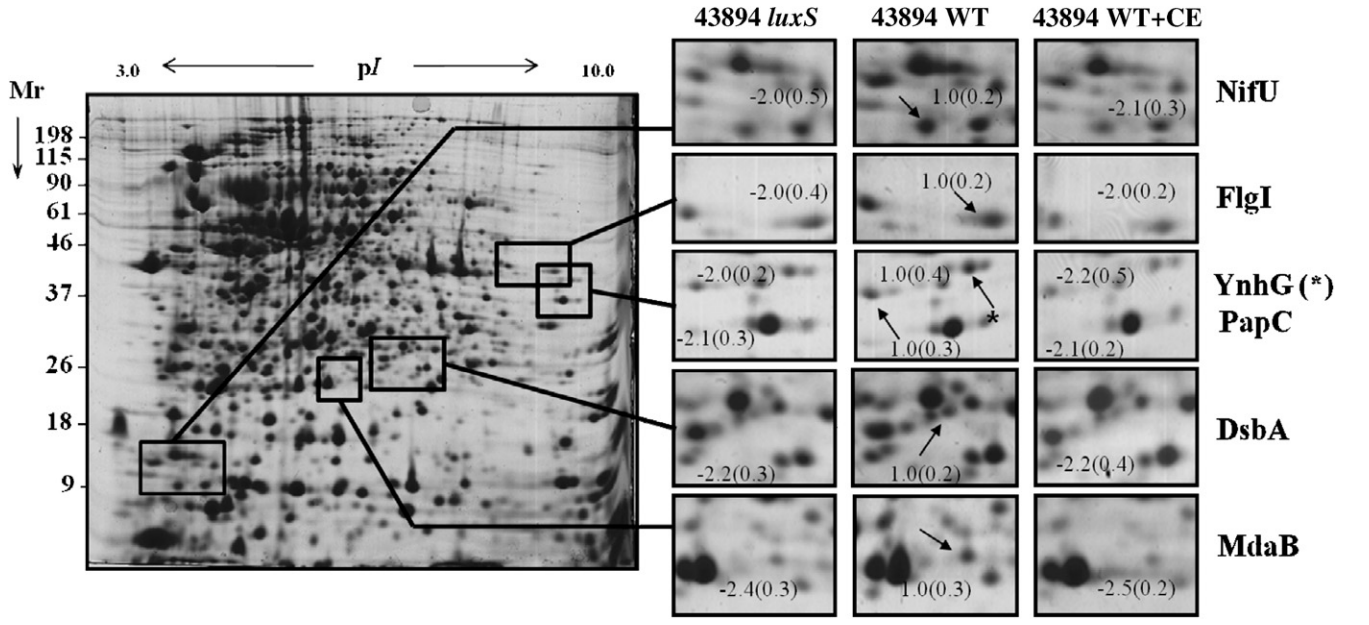

Fig. 4. Enlarged two-dimensional gel electrophoresis (2-DE) images of differentially regulated EHEC O157:H7 proteins in the presence or absence of 1.0% (w/v) cell extract isolated from *Lactobacillus acidophilus* A4. The proteins were subjected to isoelectrofocusing (pH 3–10) and resolved in the second dimension by 12.5% SDS–PAGE. The proteins were separated, detected by blue silver staining, and the data were analyzed as described in Section 2. The proteins were identified by MALDI-TOF/MS or CAF-MALDI sequencing techniques. A typical stained gel derived from EHEC O157:H7 wild type cells are shown in the left side.

consistent with *luxS*-deficient mutant, the expression of DsbA in EHEC O157:H7 were down-regulated by adding the cell extract. Recently, we observed that deletion of *dsbA* in EHEC O157:H7 resulted in loss of motility, less biofilm formation and virulence, as well as increased sensitivity to environmental stress (unpublished data; Kim and Park, 2007). Taken together, the presence of cell extracts had probably caused the reduction of DsbA, and then attenuated AI-2 like activity associated virulence phenotypes.

In addition, in the *luxS* mutant, YnhG as a novel regulon was significantly down-regulated compared to the WT strain. Consequently, in the presence of the 1.0% (w/v) cell extract the decreased expression of this protein was observed. The YnhG, which is located down stream of *sufABCDSE*, assembles iron–sulfur [Fe–S] clusters (Takahashi & Tokumoto, 2002), but its function is not clearly understood. Interestingly, this protein coupled with NifU was significantly up-regulated at the stage of *E. coli* biofilm development (Beloin et al., 2004; Schembri, Kjaergaard, & Klemm, 2003). Together with our earlier experiments, specific proteins for iron-sulfur metabolism such as NifU and YnhG may have important roles in the control of AI-2 signaling as well as the biofilm formation of EHEC O157:H7.

In conclusion, we have identified a number of active components from *L. acidophilus* A4, and have further investigated the effects of its cell extract on the inhibition of virulence development involved in attachment and biofilm formation, which is controlled by AI-2-like activity. Additional work is currently in progress to identify the active components of the cell extract, as well as to investigate the effect of the cell extract in other *in vivo* trials. Recent studies reported that several probiotic bacteria had to be alive to provoke to make an inflammatory response, whereas use of heat killed cells or its components could inhibit these responses (Choo et al., 2006; Zhang, Li, Caicedo, & Neu, 2005). Therefore, it was thought that cell extract isolated from *L. acidophilus* in this study were rational form of probiotics for the prevention of inflammation in host system. Therefore, these results indicate that newly defined cell extracts that are associated with virulence control in EHEC O157:H7 can be used as quenching agents (degradation or inactivation) of AI-2 like activity for the novel development of antibiotics and other antimicrobial agents. Our studies are currently underway to evaluate the purification and characterization of AI-2 inhibition components in the cell extracts of *L. acidophilus* A4, specifically with regard to new therapeutic strategies for inhibiting the virulence of EHEC O157:H7.

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References

Anyanful, A., Dolan-Livengood, J. M., Lewis, T., Sheth, S., Dezalia, M. N., Sherman, M. A., et al. (2005). Paralysis and killing of *Caenorhabditis elegans* by enteropathogenic *Escherichia coli* requires the bacterial tryptophanase gene. *Molecular Microbiology, 57*, 988–1007.

Beloin, C., Valle, J., Latour-Lambert, P., Faure, P., Kzreminski, M., Balestrino, D., et al. (2004). Global impact of mature biofilm lifestyle on *Escherichia coli* K-12 gene expression. *Molecular Microbiology, 51*, 659–674.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics, 77*, 71–94.

Choo, J. H., Rukayadi, Y., & Hwang, J. K. (2006). Inhibition of bacterial quorum sensing by vanilla extract. *Letters in Applied Microbiology, 42*, 637–641.

Coconnier, M. H., Klaenhammer, T. R., Kerneis, S., Bernet, M. F., & Servin, A. L. (1998). Protein-mediated adhesion of *Lactobacillus acidophilus* BG2FO4 on human enterocyte and mucus-secreting cell lines in culture. *Applied and Environmental Microbiology, 58*, 2034–2039.

Cormack, B. P., Valdivia, R. H., & Falkow, S. (1996). FACS-optimized mutants of the green fluorescent protein (GFP). *Gene, 173*, 33–38.

Daines, D. A., Bothwell, M., Furrer, J., Unrath, W., Nelson, K., Jarisch, J., et al. (2005). *Haemophilus influenzae luxS* mutants form a biofilm and have increased virulence. *Microbial Pathogenesis, 39*, 87–96.

DeLisa, M. P., Wu, C. F., Wang, L., Valdes, J. J., & Bentley, W. E. (2001). DNA microarray-based identification of genes controlled by autoinducer 2-stimulated quorum sensing in *Escherichia coli*. *Journal of Bacteriology, 183*, 5239–5547.

Gonzalez Barrios, A. F., Zuo, R., Hashimoto, Y., Yang, L., Bentley, W. E., & Wood, T. K. (2006). Autoinducer 2 controls biofilm formation in *Escherichia coli* through a novel motility quorum-sensing regulator (MqsR, B3022). *Journal of Bacteriology, 188*, 305–316.

Gram, L., de Nys, R., Maximilien, R., Givskov, M., Steinberg, P., & Kjelleberg, S. (1996). Inhibitory effects of secondary metabolites from the red alga *Delisea pulchra* on swarming motility of *Proteus mirabilis*. *Applied and Environmental Microbiology, 62*, 4284–4287.

Hentzer, M., Riedel, K., Rasmussen, T. B., Heydorn, A., Andersen, J. B., Parsek, M. R., et al. (2002). Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound. *Microbiology, 148*, 87–102.

Kanamaru, K., Kanamaru, K., Tatsuno, I., Tobe, T., & Sasakawa, C. (2000). SdiA, an *Escherichia coli* homologue of quorum-sensing regulators, controls the expression of virulence factors in enterohaemorrhagic *Escherichia coli* O157:H7. *Molecular Microbiology, 38*, 805–816.

Kim, J. U., Kim, Y., Han, K. S., Oh, S., Whang, K. Y., Kim, J. N., et al. (2006a). Function of cell-bound and released exopolysaccharides produced by *Lactobacillus rhamnosus* ATCC 9595. *Journal of Microbiology and Biotechnology, 16*(6), 939–945.

Kim, Y., Han, K. S., Imm, J. Y., Oh, S., You, S., Park, S., et al. (2006b). Inhibitory effects of *Lactobacillus acidophilus* lysates on the cytotoxic activity of shiga-like toxin 2 produced from *Escherichia coli* O157:H7. *Letters in Applied Microbiology, 43*, 502–507.

Kim, Y., Lee, Y., Kim, S. H., Yeom, J., Kim, B. S., Oh, S., et al. (2006c). Role of periplasmic antioxidant enzymes (superoxide dismutase and thiol peroxidase) of the shiga toxin-producing *Escherichia coli* O157:H7 in the formation of biofilms. *Proteomics, 6*, 6181–6193.

Kim, Y., Oh, S., Ahn, E. Y., Imm, J. Y., Oh, S., Park, S., et al. (2007). Proteome analysis of virulence factor regulated by autoinducer-2 like activity in *Escherichia coli* O157:H7. *Journal of Food Protection, 20*, 300–307.

Manefield, M., Harris, L., Rice, S. A., de Nys, R., & Kjelleberg, S. (2000). Inhibition of luminescence and virulence in the black tiger prawn

(*Penaeus monodon*) pathogen *Vibrio harveyi* by intercellular signal antagonists. *Applied and Environmental Microbiology, 66*, 2079–2084.

McNab, R., Ford, S. K., El-Sabaeny, A., Barbieri, B., Cook, G. S., & Lamont, R. J. (2003). *LuxS*-based signaling in *Streptococcus gordonii*: Autoinducer 2 controls carbohydrate metabolism and biofilm formation with *Porphyromonas gingivalis*. *Journal of Bacteriology, 185*, 274–284.

Merritt, J., Qi, F., Goodman, S. D., Anderson, M. H., & Shi, W. (2003). Mutation of luxS affects biofilm formation in *Streptococcus mutans*. *Infection and Immunity, 71*, 1972–1979.

Mylonakis, E., Ausubel, F. M., Perfect, J. R., Heitman, J., & Calderwood, S. B. (2002). Killing of *Caenorhabditis elegans* by *Cryptococcus neoformans* as a model of yeast pathogenesis. *Proceedings of the National Academy of Sciences of the USA, 99*, 15675–15680.

Oh, S., Kim, S., & Worobo, R. W. (2000). Characterization and purification of a bacteriocin produced by *Lactobacillus acidophilus* 30SC: Human isolate for potential use as a probiotic strain. *Journal of Dairy Science, 83*, 2747–2752.

Osaki, T., Hanawa, T., Manzoku, T., Fukuda, M., Kawakami, H., Suzuki, H., et al. (2006). Mutation of *luxS* affects motility and infectivity of *Helicobacter pylori* in gastric mucosa of a Mongolian gerbil model. *Journal of Medical Microbiology, 55*, 1477–1485.

Rasmussen, T. B., Bjarnsholt, T., Skindersoe, M. E., Hentzer, M., Kristoffersen, P., Kote, M., et al. (2005). Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector. *Journal of Bacteriology, 187*, 1799–1814.

Ren, D., Sims, J. J., & Wood, T. K. (2001). Inhibition of biofilm formation and swarming of *Escherichia coli* by (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone. *Environmental Microbiology, 3*, 731–736.

Ren, D., Bedzyk, L. A., Ye, R. W., Thomas, S. M., & Wood, T. K. (2004). Differential gene expression shows natural brominated furanones interfere with the autoinducer-2 bacterial signaling system of *Escherichia coli*. *Biotechnology and Bioengineering, 88*, 630–642.

Ren, D., Zuo, R., Gonzalez Barrios, A. F., Bedzyk, L. A., Eldridge, G. R., Pasmore, M. E., et al. (2006). Differential gene expression for investigation of *Escherichia coli* biofilm inhibition by plant extract ursolic acid. *Applied and Environmental Microbiology, 71*, 4022–4034.

Schembri, M. A., Kjaergaard, K., & Klemm, P. (2003). Global gene expression in *Escherichia coli* biofilms. *Molecular Microbiology, 48*, 253–267.

Sela, S., Frank, S., Belausov, E., & Pinto, R. (2006). A Mutation in the *luxS* gene influences *Listeria monocytogenes* biofilm formation. *Applied and Environmental Microbiology, 72*, 5653–5658.

Sperandio, V., Mellies, J. L., Nguyen, W., Shin, S., & Kaper, J. B. (1999). Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorrhagic and enteropathogenic *Escherichia coli*. *Proceedings of the National Academy of Sciences of the USA, 96*, 15196–15201.

Sperandio, V., Torres, A. G., Giron, J. A., & Kaper, J. B. (2001). Quorum sensing is a global regulatory mechanism in enterohemorrhagic *Escherichia coli* O157:H7. *Journal of Bacteriology, 183*, 5187–5197.

Surette, M. G., & Bassler, B. L. (1998). Quorum sensing in *Escherichia coli* and *Salmonella typhimurium*. *Proceedings of the National Academy of Sciences of the USA, 95*, 7046–7050.

Taga, M. E., Semmelhack, J. L., & Bassler, B. L. (2001). The *luxS*-dependent autoinducer AI-2 controls the expression of an ABC transporter that functions in AI-2 uptake in *Salmonella typhimurium*. *Molecular Microbiology, 42*, 777–793.

Takahashi, Y., & Tokumoto, U. (2002). A third bacterial system for the assembly of iron–sulfur clusters with homologs in archaea and plastids. *Journal of Biological Chemistry, 277*, 28380–28383.

Tan, M. W., Mahajan-Miklos, S., & Ausubel, F. M. (1999). Killing of *Caenorhabditis elegans* by *Pseudomonas aeruginosa* used to model mammalian bacterial pathogenesis. *Proceedings of the National Academy of Sciences of the USA, 96*, 715–720.

Tenor, J. L., McCormick, B. A., Ausubel, F. M., & Aballay, A. (2004). *Caenorhabditis elegans*-based screen identifies *Salmonella* virulence factors required for conserved host-pathogen interactions. *Current Biology, 14*, 1018–1024.

Xavier, K. B., & Bassler, B. L. (2003). LuxS quorum sensing: More than just a numbers game. *Current Opinions in Microbiology, 6*, 191–197.

Xavier, K. B., & Bassler, B. L. (2005). Interference with AI-2-mediated bacterial cell–cell communication. *Nature, 437*, 750–753.

Zhang, L., Li, N., Caicedo, R., & Neu, J. (2005). Alive and dead *Lactobacillus rhamnosus* GG decrease tumor necrosis factor-α-induced interleukin-8 production in caco-2 cells. *The Journal of Nutrition, 135*, 1752–1756.