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Proteome Analysis of Virulence Factor Regulated by Autoinducer-2–like Activity in *Escherichia coli* O157:H7

YOUNGHOON KIM,[1] SANGNAM OH,[2] EUN YOUNG AHN,[3] JEE-YOUNG IMM,[4] SEJONG OH,[5] SUNGSU PARK,[3] AND SAE HUN KIM[1]*

[1]*Division of Food Science, Korea University, Seoul 136-701, Korea;* [2]*Environmental Toxico-Genomic and Proteomic Center, Department of Biomedical Science, Korea University, Seoul 136-705, Korea;* [3]*Division of Nano Science, Ewha Womans University, Seoul 120-750, Korea;* [4]*Department of Food & Nutrition, Kookmin University, Seoul 136-702, Korea; and* [5]*Institute of Agricultural Science & Technology, Department of Animal Science, Chonnam National University, Gwangju 500-757, Korea*

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ABSTRACT

Many pathogenic bacteria, including *Escherichia coli* O157:H7, can control gene expression in a cell density–dependent manner by producing small signaling molecules (autoinducers) in a process known as quorum sensing. In this study, the effects of the autoinducer-2–like activity on the expression of proteins, including virulence factors, in *E. coli* O157:H7 were characterized by proteomic analysis. Compared with the control, *E. coli* O157:H7 strains in the presence of autoinducer-2–like activity exhibited elevated virulence by more rapidly forming cell aggregates on epithelial cells and rapidly killing the nematode *Caenorhabditis elegans,* the surrogate host. Two-dimensional gel electrophoresis revealed 18 proteins that were upregulated by autoinducer-2–like activity and 4 proteins that were down-regulated. These proteins were further characterized by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry and are involved in the metabolic process, adaptation and protection, cell motility, secretion, envelope biogenesis, and protein translation. These results indicate that the newly identified proteins are associated with the control of virulence in *E. coli* O157:H7 and that these proteins can be potential targets for the development of antibiotics and other antimicrobial agents.

Escherichia coli O157:H7 is an important foodborne pathogen that must be controlled in various foods to prevent foodborne illnesses, such as hemorrhagic colitis and hemolytic uremic syndrome *(21, 34).* Most prevention measures have been focused on reducing the viability of *E. coli* O157:H7, whereas a relative paucity of prevention measures have been focused on attenuating its virulence.

Quorum sensing (QS) systems, which are responsible for regulation of gene expression in response to cell population density, are widely conserved throughout the bacterial kingdom *(23, 32).* QS systems were first characterized in the marine bacteria *Vibrio harveyi* and *Vibrio fischeri (10).* In *V. harveyi,* there are two types of density-dependent signaling systems that regulate bioluminescence activity consisting of a sensor and extracellular molecules, referred to as autoinducers (AI). Some bacterial species employ their own unique signaling molecules (AI-1), a family of related homoserine lactones, for intraspecies communication, whereas other bacterial species, including *E. coli* O157:H7, use a common signaling molecule, AI-2, for interspecies communication *(31, 38). E. coli* O157:H7 modulates the expression of its virulence genes, including those associated with Shiga toxin, siderophore synthesis, motility, and biofilm formation, via the AI-2 signaling pathway *(18, 34, 35).*

Although the identification of virulence factors associated with pathogenic bacteria must be conducted in a host, in vivo trials using animals are expensive and time-consuming and require approval by ethics committees. Therefore, there is an increasing need for simple, facile, and inexpensive model organisms that can be used in the study of interactions between pathogenic bacteria and hosts *(8).* The use of the nematode *Caenorhabditis elegans* as a host model has many advantages in studies concerning the virulence mechanisms possessed by many bacterial pathogens, including *Salmonella* Typhimurium, *Pseudomonas aeruginosa, Enterococcus faecalis, Streptococcus pneumoniae,* and enteropathogenic *E. coli (1, 17).*

The application of global identification methodologies has resulted in identification of quorum-regulated processes and the characterization of the quorum circuit architecture in *E. coli, Streptococcus pneumoniae,* and *Pseudomonas aeruginosa (10, 23).* Transcriptome analysis with a DNA microarray has generated a huge amount of data on genes likely to be involved in virulence factors. However, this method provides information on the mRNA (the transmitters of genetic information) but not on the functional cellular proteins themselves. In contrast to DNA microarray analysis, proteomic studies have been used extensively for the characterization of bacterial responses to a variety of stimuli and stresses, based on levels of functional protein expression *(24, 25).* However, the response of *E. coli* O157:H7 to AI-2 signals has not yet been assessed by proteomic analysis.

In this study, we identified proteins responsible for increased attachment and virulence of *E. coli* O157:H7 in the

* Author for correspondence. Tel: +82-2-3290-3055; Fax: +82-2-3290-3506; E-mail: saehkim@korea.ac.kr.

TABLE 1. *Bacterial strains and plasmid used in this study*

Strain or plasmid	Genotype and description	Reference
Strain		
43888	*E. coli* O157:H7 (no *stx*[a])	ATCC[b]
43889	*E. coli* O157:H7 (*stx*$_2$)	ATCC
43890	*E. coli* O157:H7 (*stx*$_1$)	ATCC
43894	*E. coli* O157:H7 (*stx*$_1$ and *stx*$_2$)	ATCC
43895	*E. coli* O157:H7 (*stx*$_1$ and *stx*$_2$)	ATCC
43894-GFP	43894 with plasmid pKEN-gfpmut2	This study
JM109	*endA1 recA1 gyrA96 thi hsdR17* (*rk*−, *mk*+) *rel A1 supE44* Δ(*lac-proAB*) [*F′ traD36 proAB laqIqZΔM15*]	Laboratory stock
DH5α	*supE44* Δ*lacU159* (ϕ80 *lacZ(M15)) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory stock
OP50	Laboratory food source for *C. elegans*	*1*
BB152	*V. harveyi luxMN* (AI-1$^-$, AI-2$^+$)	*35*
BB170	*V. harveyi luxN*::Tn*5* (sensor-1$^-$, sensor-2$^+$	*35*
Plasmid		
pKEN-gfpmut2	Am^R, expression of enhanced GFP	*7*

[a] *stx,* Shiga-like toxin gene.
[b] ATCC, American Type Culture Collection (Manassas, Va.).

presence of AI-2–like activity. Phenotype changes with regard to attachment and virulence in *E. coli* O157:H7 in the presence of AI-2–like activity were first identified using in vitro and in vivo model systems. Proteins associated with elevated attachment and virulence were identified by two-dimensional gel electrophoresis (2-DE) coupled with matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

MATERIALS AND METHODS

Bacterial strains, plasmid, and culture media. Bacterial strains and the plasmid used in this study are shown in Table 1. The *E. coli* strains were cultured at 37°C in Luria-Bertani (LB) broth (Difco, Becton Dickinson, Sparks, Md.) unless otherwise stated. Attachment experiments were performed using strains harboring the green fluorescent protein (GFP) plasmid pKEN-gfpmut2 *(7)* to visualize the phenotypes of attachment in the epithelial cells. For selection of GFP-expressing strains, the medium was supplemented with 100 μg/ml ampicillin. *V. harveyi* BB152 and BB170 strains used for identification of AI-2 activity were grown aerobically at 30°C in AI bioassay medium *(10).* All other chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise stated.

Preparation of AI-like activity medium. The *E. coli* O157:H7 cultures were grown aerobically overnight in LB broth and then subcultured (inoculum of ca. 10^6 CFU/ml) into LB broth supplemented with 0.5% glucose and incubated for various times at 30°C with aeration. Conditioned media (CM) with AI-2–like activity were prepared by removing the cells from cultures via centrifugation at 12,000 × *g* for 10 min at each set time point. The cleared CM were adjusted to pH 7.5, sterilized twice with 0.22-μm-pore-size filters (Sartorious, Goettingen, Germany), and stored at −80°C. The CM from *V. harveyi* BB152 and *E. coli* DH5α were used as positive and negative controls, respectively, as previously reported *(31, 37).* In this study, the CM from *E. coli* O157:H7 strains were prepared for further experiments from culture fluids for 6 h on LB plus 0.5% glucose medium at 30°C and used for AI-2–like activity. Sterile fresh medium (0.5× LB, pH 7.5) was used as the control to investigate the effect of AI-2–like activity. Growth was monitored spectrophotometrically with a spectrophotometer (UV-1600; Shimadzu, Tokyo, Japan) by measuring the optical density at 600 nm at the same set time point.

AI-2 activity bioassay. AI-2–like activity from *E. coli* O157:H7 was determined and quantified according to previously described methods *(37)* with the *V. harveyi* BB170 reporter strain. The time course of bioluminescence was evaluated with a luminometer (Wallac model 1420 multilabel counter, Perkin Elmer, Boston, Mass.). The AI-2 activity was normalized as 100% activity by the light production of *V. harveyi* BB152 as a positive control.

Attachment assay. The HT-29 cell line was obtained from the Korea Cell Line Bank (KCLB, Seoul, Korea). The cells were routinely cultured in RPMI 1640 medium (Gibco BRL, Rockville, Md.) supplemented with 10% heat-inactivated fetal bovine serum. Before the attachment assay, the HT-29 monolayers were washed three times in prewarmed (25°C) phosphate-buffered saline (PBS) to remove any culture medium and nonattached cells. The prepared *E. coli* O157:H7 GFP strains (10^6 CFU/ml) in the presence of AI-2–like activity were then applied for 30 and 120 min at 37°C in a 5% CO_2 atmosphere. After incubation, the monolayers were washed six times with prewarmed PBS to remove nonattached bacteria. The adherent cells were released from well plates by washing with trypsin-EDTA for 5 min at 37°C. Antibiotic-free medium was then added to each of the wells and agitated to induce the dissociation of the HT-29 cells. Serial dilutions of the mixtures were then plated onto MacConkey agar (Difco, Becton Dickinson) and incubated for 48 h at 37°C. Attachment ability was determined by counting the CFU per milliliter. In analogous experiments, fluorescent images of attached *E. coli* O157:H7 43894-GFP on HT-29 cells were directly assessed with fluorescence microscopy (Olympus IX71, Tokyo, Japan) at identical incubation times as used to observe changes of attachment phenotypes in association with AI-2–like activity.

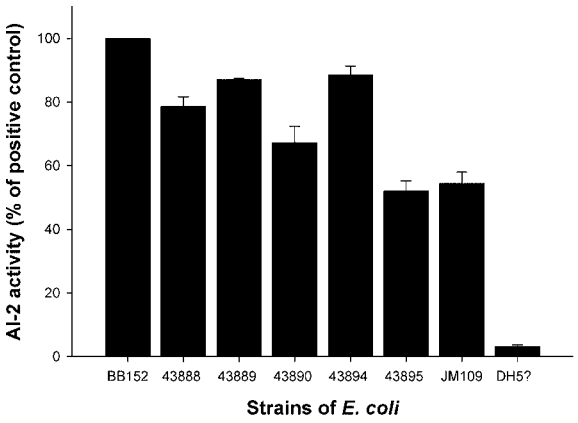
Nematode *C. elegans* killing assay. *C. elegans* killing assays were performed as initially described *(30)* with slight modification. Overnight culture samples were washed twice with PBS and resuspended in brain heart infusion (BHI). Fifteen microliters of culture was then spread on BHI agar plates (30 mm diameter) containing 20% AI-2–like activity. Following 12 h of incubation at 25°C, L4 or adult wild-type Bristol N2 worms (15 of each) were transferred to the plates, which were then sealed with parafilm and incubated at 25°C. The number of living worms per plate was determined with light microscopy (CH30 microscope, Olympus, Tokyo, Japan) at various time points for 6 days. Nematodes were considered dead when they failed to respond to tapping on the plate.

Preparation of protein samples for 2-DE. *E. coli* O157:H7 cells were grown overnight in 5 ml of LB medium. After inoculation (ca. 10^6 CFU/ml) in sterile fresh medium or AI-2–like activity medium, each of the cultures was grown for 2 h at 37°C with agitation. To improve protein focusing, the total proteins

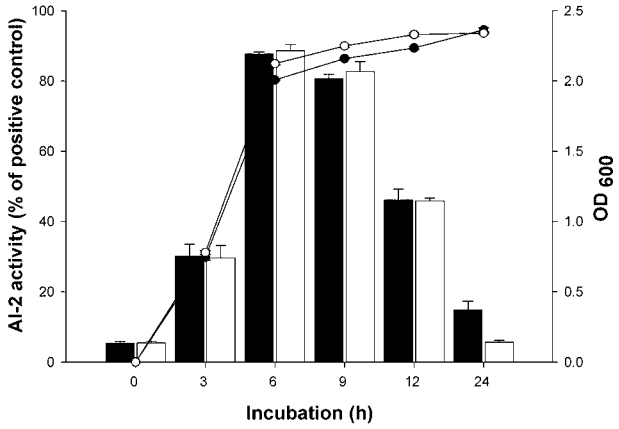
were extracted twice with phenol as previously described *(2).* Aliquots (2 ml) of each of the extracts were thawed and transferred to 15-ml polypropylene tubes. One milliliter of phenol (Sigma) was then added, and the sample was vortexed and heated for 10 min at 70°C. The sample was cooled on ice for 5 min, and the phases were separated by centrifugation for 20 min at 12,000 × *g*. The top aqueous phase was discarded, and 1 ml of distilled H_2O was added. The sample was then vortexed and heated for 10 min at 70°C. The sample was cooled on ice for 5 min, and the phases were again separated by centrifugation as before, after which the top aqueous phase was discarded. The proteins were precipitated by the addition of cold acetone for 2 h at 4°C. After centrifugation, the supernatant was poured off, and cold acetone was added. The pellet was disrupted by vigorous vortexing, and the precipitated proteins were pelleted by a final centrifugation. The supernatant was poured off, and the pellet was air dried for 2 h under a hood.

2-DE. To perform the first dimension of the electrophoresis, each of the samples was mixed with rehydration solution (8 M urea, 2% CHAPS, 50 mM dithiothreitol, 10% isopropanol, 5% glycerol, 0.5% ampholytes at pH of 3 to 10, and a trace of bromophenol blue), resulting in a final protein content of 700 μg in a total volume of 300 μl. Samples were then rehydrated on ReadyStrip IPG Strips (17 cm, pH 3 to 10, nonlinear; Bio-Rad, Richmond, Calif.) under mineral oil for 15 h at 20°C. Isoelectric focusing (IEF) was conducted using a PROTEAN IEF cell (Bio-Rad) in accordance with the manufacturer's instructions and under the following conditions: 250 V for 30 min, 500 V for 30 min, 1,000 V for 30 min, 8,000 V for 2 h, 8,000 V for 35,000 V·h, and 500 V for 15 min. After IEF separation, the strips were incubated for 10 min in equilibration buffer I (6 M urea, 2% sodium dodecyl sulfate [SDS], 20% glycerol, 130 mM dithiothreitol, and 0.375 M Tris-HCl, pH 8.8) and then for an additional 15 min in equilibration buffer II (6 M urea, 2% SDS, 20% glycerol, 135 mM idoacetamide, and 0.375 M Tris-HCl, pH 8.8). After equilibration, the strips were transferred to 12.5% SDS-polyacrylamide gels (20 by 22 cm) for the second dimension of the electrophoresis. The separation was conducted using a PROTEAN II xi system (Bio-Rad) with 10 mA per gel for 1 h and thereafter with 20 mA per gel at 4°C. The protein spots were visualized by blue-silver staining *(6).* The stained gels were then scanned with a densitometric scanner (800 by 1,600 dpi; UTA 2100XL, UMAX, Techville, Inc., Dallas, Tex.), and the spots were analyzed with PDQuest software (Bio-Rad) in accordance with the manufacturer's instructions. Four gels resulting from two independent experiments were obtained, and two gels of good quality were used for analysis. Only significant spot intensity changes (at least 2.5-fold) were considered and selected for MALDI-TOF-MS analysis.

Protein identification by MALDI-TOF-MS. The sample preparation for MALDI-TOF-MS in-gel digestion was performed using a previously described method *(27).* An Ettan MALDI-TOF (Amersham Pharmacia Biotech, Uppsala, Sweden) with an UV nitrogen laser (337 nm), delayed extraction, low-mass rejection, and a harmonic reflectron was employed in positive ion reflector mode for peptide mass mapping. Ion acceleration voltage was 20 kV. Spectra were calibrated with internal calibration using trypsin autodigestion fragments. For each spectrum, 300 single shots were accumulated. The obtained spectra were subjected to a MASCOT (http://matrixscience.com) and ProFound (http://129.85.19.192/profound_bin/WebProFound.exe) search with the *E. coli* subset of the NCBInr database for protein identification.



(A)



(B)

FIGURE 1. *Production of AI-2–like activity by various* E. coli *O157:H7 strains (A) and time profiles of AI-2–like activity produced by* E. coli *O157:H7 strains ATCC 43889 (closed symbols) and ATCC 43894 (open symbols) during 24 h of growth. AI-2–like activity in the culture fluids was determined by monitoring the induction of* V. harveyi *BB170 in a AI-2 bioassay.*

Statistical analysis. All experiments were conducted at least in triplicate. The effects of each of the treatments were evaluated with an analysis of variance followed by Duncan's test from the SAS software package (version 9.1, SAS Inc., Cary, N.C.). Significance was defined as $P < 0.05$.

RESULTS AND DISCUSSION

Production of AI-2–like activity by *E. coli* O157:H7 strains. Under CM growth conditions of 6 h in LB medium plus 0.5% glucose, AI-2–like activity produced by five *E. coli* O157:H7 strains (ATCC strains 43889, 43894, 43890, 43894, and 43895) was examined using *V. harveyi* reporter strain BB170. As expected, all the *E. coli* O157:H7 strains except DH5α induced high luminescence in *V. harveyi* BB170 (Fig. 1A). Specifically, *E. coli* O157:H7 strains 43889 and 43894 produced 88 and 89% of the normalized activity with *V. harveyi* BB152 (positive control), respectively. In addition, similar to a previous study *(37),* the production of AI-2–like activity by strains 43889 and 43894 also reached maximum levels in exponential phase (growth at 6 h) and remained high after 9 h, whereas ac-

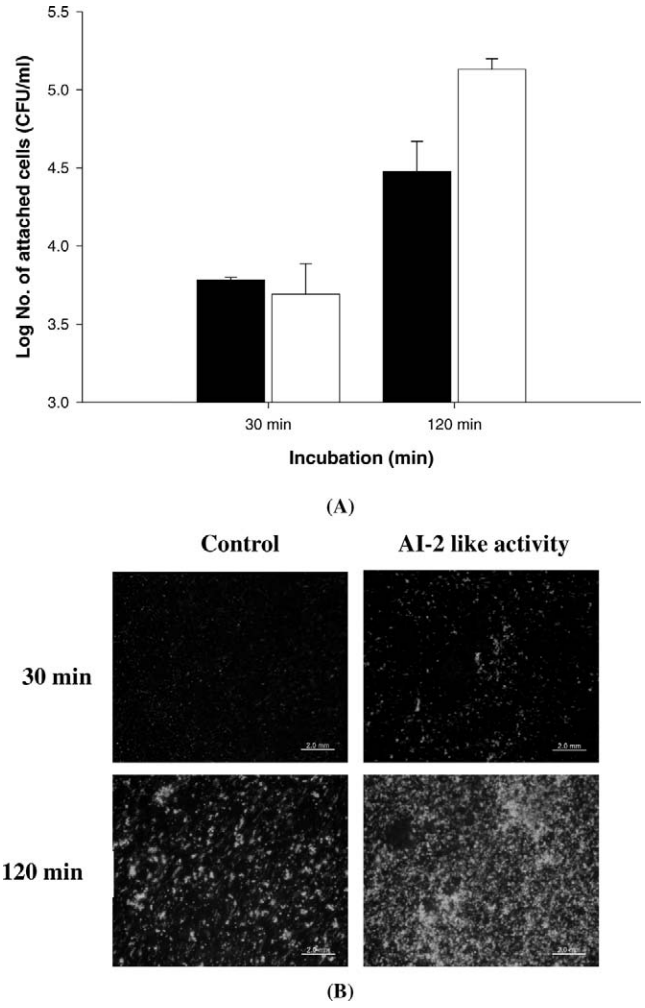


FIGURE 2. *Effect of AI-2–like activity on the attachment of* E. coli *O157:H7 ATCC 43894 to HT-29 epithelial cells (A) (closed bars, control strain 43894; open bars, strain 43894 with AI-2–like activity). Fluorescence images of* E. coli *O157:H7 43894 exposed to AI-2–like activity on HT-29 cells (B).*

tivity dramatically decreased in the stationary phase (growth at 24 h; Fig. 1B). In preliminary experiments, some virulence attributes of strain 43889 such as biofilm formation were determined to be less severe than those of strain 43894 *(20).* Therefore, in the present study, we chose to focus on strain 43894 as the host strain for examination of virulence in subsequent analyses.

Effects of AI-2–like activity on virulence of *E. coli* O157:H7. Motility and attachment are important virulence attributes for pathogens *(34).* In our preliminary experiments, in the presence of AI-2–like activity, swarm motility speed increased 1.2- to 1.4-fold at the same incubation period (8 h) compared with the control, but the results were not significantly different (data not shown). In this work, attachment experiments were performed with human epithelial HT-29 cells as an intestinal model to determine whether AI-2–like activity can increase the attaching ability of *E. coli* O157:H7. In the presence of AI-2–like activity, the attachment of *E. coli* O157:H7 43894, which is an important prerequisite for virulence manifestations, increased on HT-29 cells at 120 min compared with that in fresh

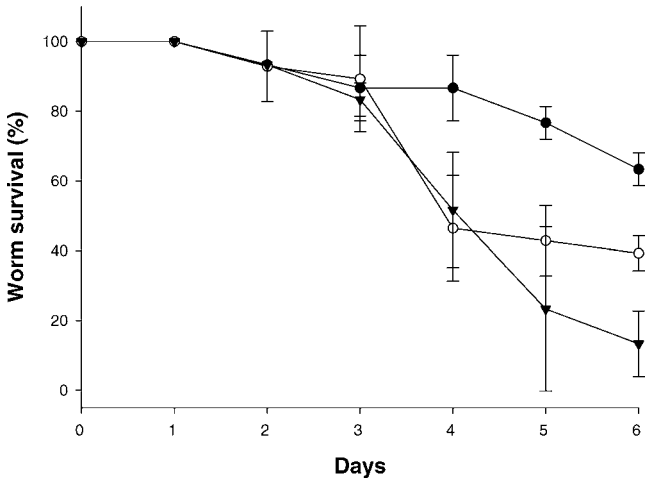


FIGURE 3. *Survival of* C. elegans *fed on* E. coli *O157:H7 ATCC 43894 in the presence of AI-2–like activity for 6 days. Closed circle,* E. coli *OP50 (laboratory food source); open circle,* E. coli *43894 (control); triangle,* E. coli *43894 (AI-2–like activity).*

medium, but there were no significant differences at 30 min (Fig. 2A). *E. coli* O157:H7 strains expressing GFP were used to visualize the attaching phenotype. Strain 43894 in the presence of AI-2–like activity more rapidly formed cell aggregates (Fig. 2B) compared with the control. These results indicate that factors related to cell aggregation and microcolony formation are regulated by AI-2 signaling. Kanamaru et al. *(18)* reported that the *E. coli* O157:H7 Sakai strain overexpressing SdiA, a QS-negative regulation factor, markedly impaired its ability to form microcolonies on Caco-2. Similar reports were found for *Pseudomonas aeruginosa (22).* Because the flagella and type III secreted proteins, including intimin and translocated intimin receptor, under the control of AI-2 signaling play an important role in the attachment of *E. coli* O157:H7 *(34, 36),* our results indicate that AI-2–like activity could act as a positive stimulator of attachment in *E. coli* O157:H7.

Although many bacterial pathogens can kill *C. elegans,* few data have been reported for *E. coli* O157:H7 *(1).* In the present work, there were no significant differences in survival rates of *C. elegans* for 5 days, but the survival of the nematodes on a plate containing 20% AI-2–like activity were lower than those on fresh medium at 6 days (Fig. 3). Previous data *(30)* have indicated that a QS-deficient mutant of *P. aeruginosa* killed fewer nematodes than did a *P. aeruginosa* wild-type strain. These data provide some evidence that the death of *C. elegans* in the presence of AI-2–like activity may be caused by induction of virulence factors. In this study, we used solid type *C. elegans* models with agar, with the addition of 20% AI-2–like activity, instead of whole AI-2–like activity as in previous experiments. At present, ongoing studies are evaluating the full induction of cytotoxicity from AI-2–like activity using liquid type *C. elegans* in vivo models as described by Jansen et al. *(17).* Therefore, all of these results support that AI-2–like activity is an important regulation factor for enhancing the virulence of *E. coli* O157:H7, as indicated by attachment to HT-29 cells and killing of *C. elegans.*

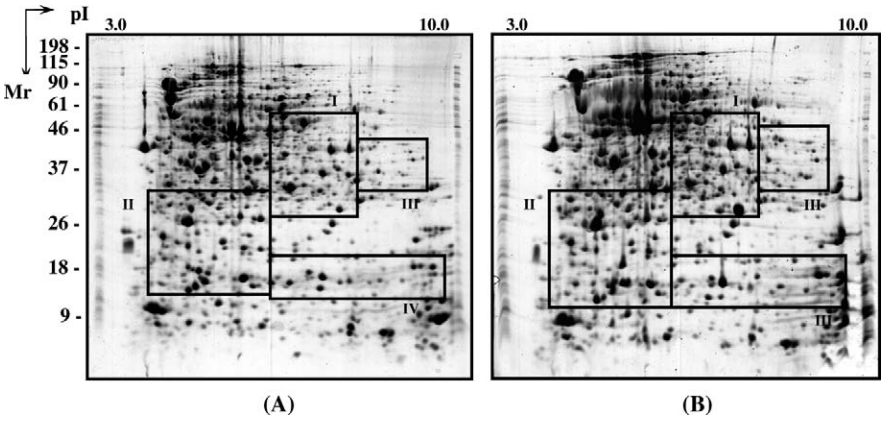


FIGURE 4. *Two-dimensional gel electrophoresis images of protein extracts of* E. coli *O157:H7 ATCC 43894 exposed to control conditions (A) and AI-2–like activity (B). The crude protein extracts (700 μg) were separated on nonlinear immobilized pH gradient strips (pH 3.0 to 10.0) and electrophoresed on a 12.5% SDS-polyacrylamide gel. Outlined sections indicate zones presented in Figure 5.*

Identification of protein regulated by AI-2–like activity. There have been no reports on the effect of AI-2 on *E. coli* O157:H7 as evaluated by 2-DE. To analyze the AI-2–like activity regulon of *E. coli* O157:H7, we used 2-DE techniques to compare the protein expression pattern of *E. coli* O157:H7 43894 grown in the absence or presence of AI-2–like activity. A total of 600 protein spots were visualized by CBB G-250 staining (Fig. 4). A total of 22 protein spots with a 2.5-fold increase or decrease in amounts were detected from *E. coli* O157:H7 43894 in the presence of AI-2–like activity compared with the control (18 and 4 spots were up- and down-regulated, respectively). The proteins identified by MALDI-TOF-MS were classified into five groups based on their functions (Table 2 and Fig. 5).

Group I proteins encode regulators for metabolic processes such as nucleotides and amino acids and iron metabolism or biosynthesis. Iron metabolism in pathogenic bacteria is of interest because iron acquisition and its associated metabolism often are related to virulence in pathogenic organisms *(28).* Spots 1 and 19 were identified as *nifU*-like protein (*nifU*) and cysteine desulfurase (*nifS*). Unfortunately, there are no previous reports on the interactions between *nifU*-like and *nifS* protein and QS signals, including AI-2. Nishio and Nakai *(26)* proposed that *nifU* serves as a scaffold for iron-sulfur cluster assembly and functions as a mediator for iron-sulfur cluster delivery. Both *nifU* and *nifS* were significantly overexpressed in mature biofilms of *E. coli* K-12 *(4).* Spot 13 was identified as copper homeostasis protein (*cutC*). Copper is critical for the proper function of several enzymes, including cytochrome oxidase and superoxide dismutase (SOD). This copper homeostasis protein plays an important role in reduction of subtoxic materials in prokaryotic cells and in the survival of pathogenic bacteria within mice and humans *(33).* There are many reports that mutations in the genes encoding for copper homeostasis decrease the pathogenicity of *P. aeruginosa (33)* and *Listeria monocytogenes (13)* in vivo trials. *cutC* also increases regulation of *E. coli* K-12 biofilm culture mode *(4).* The upregulation of active metabolic processes suggests that exposure of *E. coli* O157:H7 AI-2 increases the potential metabolic ability for limited energy and iron sources.

Group II included protein spots 2, 4 (and 12), 11, and 21, representing proteins involved in adaptation and protection. In some organisms, various changes of phenotype by QS signals are coupled with elevated resistance to a wide variety of structurally and functionally distinct classes of antimicrobial compounds *(19).* In our results, the expression of Mn-SOD (*sodA,* spots 4 and 12 with a >5-fold overexpression compared with the control) was dramatically increased. However, in two previous studies *(10, 31)* of the effect of AI-2 on *E. coli* gene expression, Mn-SOD results were very different (microarray data in Table 2).The discrepancy between these two studies could be due to differences in growth conditions, strains (*E. coli* K-12 versus *E. coli* O157:H7), and detection techniques (e.g., DNA microarray versus 2-DE). In *P. aeruginosa,* the expression of Mn-SOD was particularly dependent on N-acyl-homoserine-lactones (AI-1) *(16).* In group II, the expression of *uspG* (or *ybdQ,* spot 10) related to the universal stress protein family (putative substrates of the chaperon GroEL) *(5)* was decreased in the presence of the AI-2 signals. Similarly, a universal stress protein family of *Porphyromonas gingivalis* was down-regulated by QS signals *(42).* In stress gene mutants of *E. coli* K-12, a relationship between several stress and starvation genes and the *luxS* gene also has been found *(10).* The findings of previous studies and of our analyses suggest that some stress responses of *E. coli* O157:H7 are controlled by AI-2–like activity.

Group III contained motility and membrane proteins (spots 6, 7, 14, 15, 21, and 22) involved in secretion, transport, and envelope biogenesis. Bacterial virulence factors are typically surface-associated or secreted molecules that in gram-negative bacteria must cross the outer membrane *(38).* In *E. coli,* the flagellum also is important in the initial adhesion phase and in spreading along the surface *(29).* In this study, expression of *flgI* (spot 22) increased compared with that of the control. Elevated expression of this gene by AI-2 signaling also was reported in a previous DNA microarray study *(31).* In other studies, expression and assembly of flagellum-related genes in *E. coli* O157:H7 *(35)* and *Salmonella (14)* were upregulated by AI-2 signaling. Spot 14 was identified as DNA-binding protein (*stpA*). The *E. coli stpA* gene encodes a 15.3-kDa protein that is 58% identical to H-NS, a global gene regulator *(3, 39),* at the amino acid level. Spot 21 was identified as *dsbA,* a periplasmic disulfide-bond oxidoreductase. Dailey and Berg *(9)* reported that a *dsbA* mutant of *E. coli* was not able to form

TABLE 2. *Identification of AI-2–like activity regulated proteins by MALDI-TOF-MS peptide mass mapping*

Spot no.	Gene name	Protein identification or functions	pI[a]	M_r (Da)[a]	Sequence coverage (%)[b]	Regulation[c]	DNA microarray[d] DeLisa et al. *(10)*	DNA microarray[d] Ren et al. *(31)*	Accession no.[e]
Group I (Metabolic process)									
1	*nifU*	Formation of [Fe-S] iron-sulfur proteins	4.8	14,000	30	↑ (+3.3)	ND	ND	P77310
13	*cutC*	Copper homeostasis protein	6.7	15,732	13	↑ (+2.9)	±	ND	Q8XCH4
18	*pheS*	Phenylalanine tRNA synthetase alpha-subunit	5.8	36,840	43	↑ (+2.8)	±	ND	P67037
19	*nifS*	Cysteine desulferase	5.9	45,240	39	↑ (+3.0)	ND	ND	P0A6B9
20	*pyrB*	Aspartate carbamoyltransferase catalytic chain	6.1	34,470	44	↑ (+2.6)	±	ND	P0A788
Group II (Adaptation and protection)									
2	*terC*	Putative tellurium resistance protein C	4.6	20,540	59	↑ (+3.0)	ND	ND	Q8X9Q1
4, 12	*sodA*	Superoxide dismutase (Mn)	6.4	23,310	27	↑ (+9.5)	↑ (+1.5)	↓ (−2.8)	P66828
11	*mdaB*	Modulator of drug activity B	5.8	21,870	35	↑ (+2.6)	↑ (+2.0)	ND	P40717
10	*uspG*	Universal stress protein G	5.9	15,920	69	↓ (−3.0)	ND	ND	Q8XBT3
Group III (Cell motility, secretion, and envelope biogenesis)									
6	*papC*	Membrane-bound ATP synthase gamma-subunit	9.0	31,560	38	↑ (+3.4)	↑ (+1.6)	ND	P00837
7	*ompX*	Outer membrane protein	5.0	16,340	30	↑ (+3.3)	↑ (+1.5)	ND	P0A919
14	*stpA*	DNA-binding protein; H-NS–like protein	7.9	15,338	38	↑ (+2.5)	ND	ND	P30017
15	*ybeJ*	Putative periplasmic binding transport protein	8.8	33,520	35	↓ (−3.0)	±	ND	P37902
21	*dsbA*	Thiol:disulfide interchange protein	5.9	23,200	29	↑ (+2.6)	±	ND	P24991
22	*flgI*	Flagellar P-ring protein	9.7	38,280	33	↑ (+2.5)	±	↑ (+3.1)	P58203
Group IV (Protein translation)									
5	*rplJ*	50S ribosomal subunit protein L10	9.0	17,750	43	↑ (+6.6)	↑ (+1.5)	ND	P0A7J5
8	*rpsF*	30S ribosomal subunit protein S6	5.1	15,378	13	↑ (+4.3)	↑ (+1.9)	ND	P0A4D1
17	*rplI*	50S ribosomal subunit protein L9	6.1	15,750	53	↑ (+2.6)	±	ND	P0A7R3
Group V (Unknown functions)									
3	*hybE*	Hydrogenase-2 operon protein	5.5	17,908	17	↓ (−3.6)	±	ND	Q8XBW0
9	*ynaF*	Putative filament protein	5.8	16,000	26	↓ (−2.9)	±	ND	NP310024
16	*yeaR*	Hypothetical protein	6.0	13,620	32	↑ (+4.2)	±	ND	P64489

[a] Theoretical isoelectric point and relative molecular mass.
[b] Sequence coverage provides an indication of confidence of identification.
[c] Up- (↑) or down-regulation (↓) in the presence of AI-2–like activity.
[d] Comparison of our data with previously published *E. coli* K-12 microarray studies. ND, not determined; ±, not changed.
[e] Accession no. refers to the SWISS-Prot/TrEMBL databases.

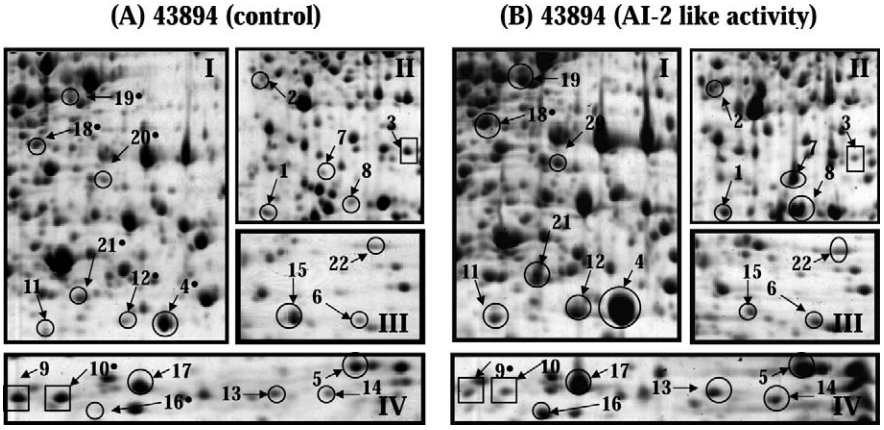


FIGURE 5. *Enlarged partial two-dimensional images representing crude protein extracts of* E. coli *O157:H7 ATCC 43894 exposed to control conditions (A) and AI-2–like activity (B). Circled and boxed protein spots indicate proteins that are up- and down-regulated by AI-2–like activity compared with controls, respectively. Marked protein spots were identified by MALDI-TOF-MS (see Table 2).*

a disulfide bond in the flagellum P-ring protein (FlgI). In *P. aeruginosa, dsbA* was essential for multiple virulence factors combined with the expression of a type III secretion system and for motility *(3, 15, 40).*

Group IV included ribosomal proteins (spots 5, 8, and 17), which are general sensors for heat and cold shock *(41).* Because the proteins in groups III and IV are thought to be sensors of various stresses and stimuli, they may be targets of special interest in proteome analyses with respect to stress response against harmful conditions generated by antimicrobial treatment and food processing *(11, 12, 41).*

Group V proteins produced by *hybE* (spot 3), *ynaF* (spot 9), and *yeaR* (spot 16) were identified previously, but no effect on the virulence of *E. coli* O157:H7 has been reported until now. Because of limited techniques, we were unable to observe the regulation of highly hydrophobic proteins from membranes and extracellular proteins from culture medium with AI-2–like activity.

Our 2-DE techniques revealed that 22 proteins of *E. coli* O157:H7 were differentially regulated in the presence of AI-2. To our knowledge, this report is the first on investigation with 2-DE of effects of AI-2–like activity on *E. coli* O157:H7 stress responses. Significant changes in expression of the proteins had not been reported in previous transcriptome-based studies *(34, 35)* probably because of the differences in detection methods (DNA microarray versus 2-DE). Most of the proteins found in our study are related to general stress responses. Some of the proteins seemed to play important roles in increasing the attachment ability and virulence of *E. coli* O157:H7, as indicated by experimental results performed with human epithelial HT-29 cells and *C. elegans* as intestinal in vitro and in vivo models, respectively.

Many proteins identified in this study were also upregulated on biofilm cultures, as predicted. In some studies, interaction between AI-2–like activity and biofilm formation in pathogenic bacteria, directly or indirectly, have been reported *(11, 30).* At present, we are using various abiotic or biotic surfaces to investigate the effects of the AI-2 signaling system on the biofilm produced by *E. coli* O157:H7.

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