J. Microbiol. Biotechnol. (2010), **20**(2), 415–424
doi: 10.4014/jmb.0904.04007
First published online 29 December 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Omics-Based Analysis of the *luxS* Mutation in a Clinical Isolate of *Escherichia coli* O157:H7 in Korea

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Received: April 8, 2009 / Revised: August 18, 2009 / Accepted: September 18, 2009

The purpose of this study was to investigate the relationship between the global regulatory mechanism known as quorum sensing and expression of virulence factors in *Escherichia coli* O157:H7. A nonpolar *luxS* deletion was introduced into the chromosome of strain CI03J, a human clinical isolate from South Korea, to create the Δ*luxS* mutant strain ML03J. Phenotypic characterization of wild-type and mutant strains demonstrated that ML03J had no obvious growth or metabolic defects on 0.2% glucose LB medium, produced a functionally defective flagellum, and could not utilize sorbose; the biological significance of sorbose utilization is unknown. Omics-based analysis revealed the involvement of LuxS in the transcriptional activation of several flagella/chemotaxis-related genes (*flhD*; *fliA*, *C*, *D*, *S*, *Z*; and *cheA*, *Y*, *Z*), repression of glutamate-dependent acid resistance genes (*gadAB*), and expression of virulence factors including Shiga toxin, hemolysin, and SepD within the LEE pathogenicity island.

Keywords: *Escherichia coli* O157:H7, clinical isolates, Korea, *luxS* mutation, omics

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 was first independently identified in 1983 as an emerging human bacterial pathogen in investigations of an outbreak of hemorrhagic colitis [24, 35] and sporadic cases of hemolytic uremic syndrome [17]. Since then, epidemic or sporadic outbreaks have been reported from all over the world; the majority have been linked with dairy products or farm environments [36]. A recent study in the United States reported that the annual incidence of EHEC O157:H7 has been reduced from 73,480 cases in 1997 to 56,911 in 2005, and its annual cost in 2005 was estimated at $333.5 million [10]. The bacterium possesses three well-defined virulence factors; Shiga toxins (Stx), a 60-MDa virulence plasmid (pO157), and the locus of enterocyte effacement (LEE) pathogenicity island [36]. In addition, EHEC O157:H7 has a very low infectious dose (estimated to be as few as 50 colony forming units) [36], which makes it a serious public health threat, although the mechanism underlying this unique property is unclear.

Bacterial virulence expression is controlled by quorum sensing (QS), a universal adaptive response triggered by small signaling molecules called autoinducers (AIs) [27, 28]. AIs are secreted and accumulate outside bacteria as the bacterial population increases. Four different bacterial AI molecules have been reported: AI-1, AI-2, AI-3, and AI-peptide-like streptococcal pheromones [34]. Among these, AI-2 and AI-3 mediating QS signaling have been extensively studied in EHEC O157:H7 [34]. The synthesis of AI-2 requires a series of biochemical conversions of *S*-adenosylmethionine to homocysteine, which are catalyzed by two enzymes, designated Pfs and LuxS [4]. However, LuxS function seems not to be limited by the extent of AI-2 production, because increasing evidence suggests that mutation in *luxS* manifests as pleiotropic phenotypes [28], some of which are not restored by the simple addition of purified AI-2, indicating an AI-2-independent gene regulation by LuxS [18]. Such phenotypes include bacterial swarm motility and LEE-encoded gene expression [18]. Indeed, recent studies have demonstrated that LuxS is indirectly involved in the production of another signaling molecule, AI-3, which is structurally distinct from AI-2 and functionally responsible for the QS-dependent motility

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and *LEE* gene expression [18, 33]. Interestingly, both AI-2 and AI-3 are universal to many bacterial species, and AI-3 and the mammalian hormones epinephrine and norepinephrine are recognized by the same receptor(s) in the QseBC and/or QseEF two-component systems [14]. Thus, these signaling molecules are important for both inter-species and inter-kingdom communication, although the biological significance of both AI-2 and -3 is not clearly understood.

Previously, transcriptomics and proteomics were used to understand LuxS/AI-2 mediated signaling in EHEC O157:H7 using either a defined *luxS* mutant strain (VS94), a derivative of EHEC O157:H7 86-24, or *in vitro* purified/synthesized AI-2-molecules [2, 18, 26]. However, variable results were obtained, which may have depended on the culture conditions (*i.e.*, culture media, growth phase, or anaerobiosis) and the isolation of the strains from different sources. Presently, we introduced a defined nonpolar *luxS* deletion into the chromosome of the strain CI03J, an EHEC O157:H7 human clinical isolate from South Korea obtained in 2003 (Kim and Cho, unpublished observations), with the aim of investigating the effect of *lux*S-mediated QS on phenotypes related to EHEC O157:H7 virulence and infection. Whole genomic expression profiles in both wild-type and isogenic Δ*luxS* mutant strains of CIO3J were determined using omics-based analyses.

Materials and Methods

Bacterial Strains and Plasmids

The bacterial strains and plasmids used are listed in Table 1. All *E. coli* strains were grown at 37°C with aeration at 200 rpm in Luria–Bertani (LB) medium containing 0.2% (w/v) glucose. When necessary, the particular antibiotics were added into the media at the following concentrations: ampicillin (Ap), 200 μg/ml; kanamycin (Km), 50 μg/ml; and chloramphenicol (Cm), 30 μg/ml.

Construction of the *E. coli* O17:H7 Δ*luxS* Mutant Strain

An isogenic Δ*luxS* mutant strain was constructed from a Korean isolate of *E. coli* O157:H7, CI03J, by a polymerase chain reaction (PCR)-based linear transformation technique [5, 8]. The plasmid pKD13 (Table 1) [8] was used as a template to amplify the Km gene cassette using gene-specific primers (F: 5'-GCG GTG CGC ATC AAG TAC AAC TAA GCC AGT TCA TTT GGT GTA GGC TGG AGC TGC TTC-3' and R: 5'-TGC GGT GTG GCT GGA AAA ACA CGC CTG ACA GAA AAG ATT CCG GGG ATC CGT CGA CC-3'). The resulting Δ*luxS* deletion was first detected by PCR using the gene-specific primers adjacent to the intact *luxS* gene (F: 5'-CGC GAG GCG TCT GAA CGC-3' and R: 5'-GGA TGA CGC AAC AGC AGG-3'). The mutated Δ*luxS* region was subcloned using the RBC T&A cloning system (Real Biotech Corporation) and confirmed by DNA nucleotide sequencing (Macrogen; data not shown).

Bacterial Growth and Swarm Motility

The wild-type and mutant strains were precultured overnight in LB broth containing 0.2% glucose at 37°C with aeration. The bacteria were diluted 1:1,000 in fresh LB medium, and the optical density at 600 nm (OD_{600}) was measured every hour to monitor bacterial growth. At least three independent experiments were performed and the data are represented as the average mean ± standard error (SE). To investigate bacterial swarm motility, *E. coli* was stabbed into either Dulbecco's modified Eagle's medium (DMEM) or tryptone agar plates containing 0.3% (w/v) Bacto agar (both from Difco) and incubated at 37°C. The diameter of each motility halo was measured at 3, 18, and 24 h.

Transmission Electron Microscopy (TEM)

Samples on glow discharged, Formvar-coated grids were prepared for TEM by negative staining using 3% (v/v) uranyl acetate. Five ml of bacterial culture was centrifuged at 10,000 rpm for 1.5 min and the pellet was resuspended in 1 ml of 50 mM KH_2PO_4 buffer. Ten μl of the bacterial suspension was applied to the grid surface and sedimented for 2 min. Then, 5 μl of 3% (v/v) uranyl acetate was applied for 1 min and excess liquid was removed using filter paper. TEM involved a JEOL JEM1010 instrument at a voltage of 80 kV.

Utilization of Carbohydrates or Amino Acids

A single colony was recovered and used to inoculate LB broth containing 0.2% (w/v) glucose. Following growth at 37°C overnight, the utilization of 49 carbohydrates or 24 amino acids was determined using appropriate assay kits (HIMEDIA Laboratories) following the manufacturer's protocols.

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

Strain/plasmid	Description	Reference
Top10	General cloning vector	Invitrogen
CI03J	Clinical isolate of *E. coli* O157:H7 in Korea (stx_1^+/stx_2^+)	This study
ML03J	Isogenic *luxS* deletion mutant of CI03J	This study
RL03J	ML03J complemented by pEXP5-CT/luxS	This study
pKD 13	Template plasmid carrying a Km^R cassette (Ap^R, Km^R)	[15]
pKD 46	Arabinose-inducible lambda red recombinase expression plasmid (Ap^R)	[15]
pCP20	Temperature-sensitive plasmid carrying a FLP flipase (Ap^R, Cm^R)	[16]
pEXP5-CT	IPTG-inducible C-terminal polyhistidine (6×His) expression vector (Ap^R)	Invitrogen
pEXP5-CT/luxS	pEXP5-CT carrying the intact *luxS* gene from CI03J (Ap^R)	This study

Transcriptome Analysis

Bacterial strains were routinely grown in LB broth containing 0.2% (w/v) glucose at 37°C with aeration. To monitor bacterial growth, OD_{600} was measured by a GeneQuant Pro spectrophotometer (GE Healthcare). Total RNA from the early stationary phase culture (approximately 0.8 OD_{600}) was stabilized by adding RNA protection reagent (Qiagen) and was isolated using a RNeasy mini kit (Qiagen) following the manufacturer's instructions. The resulting crude RNA was treated with RQ1 RNase-free DNase I (Promega) to eliminate all contaminating DNA. *E. coli* O157:H7-specific whole genome-scale microarray analysis was performed using a GenePlorer TwinChip (*E. coli*-6K format; Digital Genomics).

Proteome Analysis

E. coli cultured as described above was pelleted by centrifugation at 5,500 ×*g* for 10 min, 4°C. The resulting supernatant was filtered through a 0.22-μm syringe filter (Millipore), whereas the pellet was washed three times using phosphate-buffered saline (PBS). For the extracellular proteins (ECPs), each filtered supernatant was precipitated by trichloroacetic acid (TFA) as previously described [20]. The precipitated proteins were collected by centrifugation at 15,000 rpm, 4°C, for 20 min, washed twice with prechilled acetone at −20°C, and air-dried. ECPs were solubilized in ReadyPrep reagent 3 (5 M urea, 2 M thiourea, 2% CHAPS, 2% SB 3–10, 40 mM Tris, and 0.2% Bio-Lyte 3/10 ampholyte; Bio-Rad) and stored at −20°C until analyzed.

For the total cellular proteins (CPs), the washed pellets were resuspended in lysis buffer (40 mM Tris-HCl, 1% Triton X-100, 1 mM $MgSO_4 \cdot 7H_2O$, 1 mM EDTA, pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride. The cells were disrupted on ice by sonicating five or six times for 10 s each time with 80% pulse duration until clear lyates were obtained. The protein concentration was determined by a protein assay kit (Bio-Rad) with bovine serum albumin as a standard.

Isoelectric focusing was carried out using a linear Immobiline IPG gel (13 cm, pH 4–7; Amersham Biosciences). A total of 100 μg of ECP or CP samples were analyzed with 76,500 Vh (voltage/h) at 20°C in an IPG phor (Amersham Biosciences), wherein the voltage was linearly increased from 500–3,500 V over the first 5 h and then maintained at 3,500 V during the final 17.5 h using an EPS 3,500 XL power supply (Amersham Biosciences). Equilibration was carried out as described previously [11]. After equilibration, each IPG gel was transferred to the top of a 12% sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE) gel and pressed firmly to the slab gel surface to ensure successful subsequent protein transfer. SDS–PAGE was performed in a Pretean II xi Multi-Cell (Bio-Rad) at 40 mA per gel and the proteins were visualized using a PlusOne silver stain kit (Amersham Biosciences).

Protein Identification

Sample preparation for matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI–TOF/MS) was performed using a previously described method [23]. Briefly, protein spots of interest were excised and destained by washing with 100 mM sodium thiosulfate containing 30 mM potassium ferricyanide. The gels were dehydrated by adding 100% acetonitrile (ACN), and rehydrated in ice by adding 50 μl of 200 mM ammonium bicarbonate containing 10 mg/ml of sequencing-grade-modified trypsin (Promega). After incubation at 37°C for 20 h, the peptides were extracted with 0.1% TFA in 50% ACN. The supernatants were recovered and vacuum-dried. The samples were reconstituted in 0.1% TFA and concentrated with C18 ZipTips (Millipore). The purified peptides were eluted with a saturated matrix solution (α-cyano-4-hydroxycinnamic acid in 60% ACN and 0.1% TFA). The monoisotopic masses (M+1) of the trypsinized fragments were measured in a Perspective Biosystem MALDI–TOF/MS voyager DE-STR mass spectrometer (Framingham). The spectra were searched and identified using the MS-Fit system (http://prospector.ucsf.edu/prospector/4.0.8/html/msfit.htm) with an *E. coli* subset of the National Center for Biotechnology Information database.

Cytotoxicity

The water-soluble tetrazolium salt (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium, monosodium salt) (WST) was used to measure Vero cytotoxicity of the wild-type and Δ*luxS* mutant strains. The WST-1 based assay was carried out using an EZ-CyTox kit (iTSBio) following the manufacturer's description. Stx-producing *E. coli* O157:H7 ATCC43895 and *E. coli* JM109 strains were used as the positive and negative controls, respectively, for all assays.

Statistical Analyses

All experiments in the study were conducted on at least three independent experiments in triplicate. The effects of each treatment were analyzed by ANOVA, followed by Duncan's test using SAS Version 9.1 (SAS Inc.). The level of significance was defined at $p<0.05$. For the microarray data, hybridization images were analyzed by GenePix Pro 3.0 software (Axon Instrument) to obtain gene expression ratios (reference versus test samples). Gene expression ratios were normalized by GenePix Pro 3.0 software. Clustering images were obtained from hierarchical clustering [9], which included computing "distances" between data elements.

RESULTS

Defined Deletion of the Chromosomally Encoded *luxS* Gene in CI03J

To investigate the effect of *luxS* mutation in CI03J, a human clinical isolate of *E. coli* O157:H7 obtained in South Korea was used as the basis for the construction of an isogenic Δ*luxS* mutant strain missing the entire structural *luxS* gene by a standard one-step gene inactivation technique [5, 8]. Defined genomic deletion of the *luxS* gene was detected by PCR (Fig. 1A) and further confirmed by DNA nucleotide sequencing (data not shown). As shown in Fig. 1B, DNA sequencing analysis revealed that the 698-bp genomic region containing both 164-bp upstream and 20-bp downstream nucleotides of the intact structural *luxS* gene in *E. coli* O157:H7 CI03J was deleted and replaced by the 81-bp scar nucleotides originating from the template plasmid pKD13, where all three forward stop codons but no translation signals have been reported [8]. The resulting Δ*luxS* mutant strain of CI03J was designated as ML03J. For the complementation assays, the isopropyl-β-D-thiogalactopyranoside-inducible expression plasmid carrying

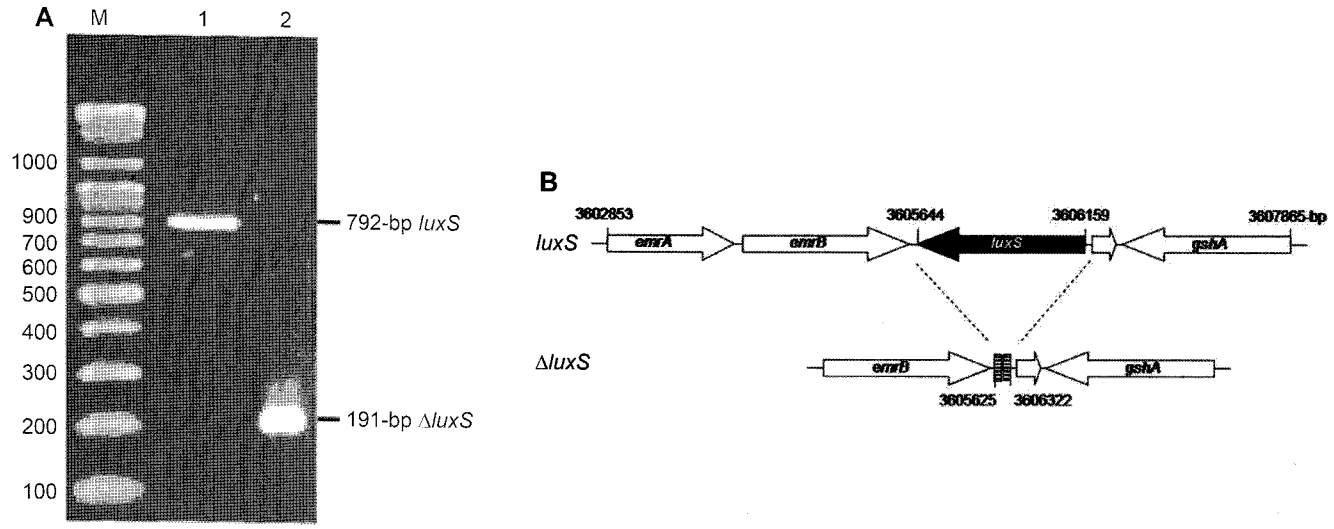

Fig. 1. Defined genetic mutation of the *luxS* gene in CI03J, a clinical isolate of *E. coli* O157:H7 from Korea.
A. *luxS* deletion was confirmed by PCR using the gene-specific primers adjacent to the *luxS*. The PCR products were separated on 1% agarose gel. Lane M, 1.0-kb plus DNA ladder (Invitrogen); lane 1, CI03J (wild type); lane 2, ML03J (CI03J Δ*luxS*). **B.** Schematic genetic map of the defined Δ*luxS* region in ML03J. The DNA sequence from the Δ*luxS* region was compared with the genomic DNA sequence of *E. coli* O157:H7 EDL933 (GenBank Accession No. AE005174). The striped boxes indicate the 81-bp scar nucleotide sequence in the Δ*luxS* mutant strain ML03J originated from the template plasmid pKD13.

the intact *luxS* gene (pEXP5-CT/luxS) was created and transformed into the mutant strain ML03J. The resulting complemented strain was designated RL03J and all three strains (CI03J, ML03J, and RL03J) were used for further analyses (Table 1).

ML03J Displays No Growth or Metabolic Defects in LB Medium Containing 0.2% Glucose, but Cannot Utilize L-Sorbose

To test if the *luxS* mutation resulted in any metabolic burdens in *E. coli* O157:H7, we monitored bacterial growth in nutrient-rich LB medium containing 0.2% glucose as well as examined the capability of the bacteria to utilize various carbohydrates and amino acids. As shown in Fig. 2A, the Δ*luxS* mutant strain ML03J showed growth kinetics similar to the wild-type strain CI03J in 0.2% glucose-containing LB medium, indicating no obvious growth and/or metabolic defects attributable to Δ*luxS* mutation. Supporting this notion, the Δ*luxS* mutant strain ML03J showed the same pattern as the wild-type strain CI03J in the ability to utilize almost all the carbohydrates and amino acids (Tables S1 and S2). An exception was seen with L-sorbose. The Δ*luxS* mutant strain ML03J was not able to utilize L-sorbose, unlike the wild-type strain CI03J (Table S1).

Unlike in 0.2% glucose LB media, however, the Δ*luxS* mutant strain ML03J showed a slight growth defect compared with the wild-type strain CI03J when grown in DMEM, a virulence-inducing minimal medium for *E. coli* O157:H7 [19] (Fig. 2B). The observed slowed growth by Δ*luxS* mutation has been previously reported in *E. coli* O157:H7 strain 86-24 [28].

ML03J is Less Motile Than the Wild-Type Strain

To compare flagellar biosynthesis and flagella functionality in the wild-type and Δ*luxS* mutant strains, the bacterial flagellum was directly visualized by TEM, and functionality was assessed using 0.3% tryptone agar or DMEM. TEM analyses of the wild-type, Δ*luxS* mutant, and complemented RL03J strains uniformly revealed flagella (Fig. 3A). The result was unexpected because previous studies demonstrated that *luxS* mutation causes repression of several genes involved in flagella biosynthesis [6, 7, 28, 30]. Therefore, we hypothesized that the flagellum of the Δ*luxS* mutant strain might not be fully functional or that the Δ*luxS* mutant strain has a reduced number of flagella compared with the wild-type strain CI03J. To test this hypothesis, bacterial swarm motility was examined. As expected, ML03J was less motile than the wild-type strain CI03J in both 0.3% tryptone and DMEM agar plates (Fig. 3B). In contrast, the complemented strain RL03J restored swarm motility in both media (Fig. 3B).

Transcriptome Profiling Identification of 35 *E. coli* O157:H7 Genes Differentially Regulated by at Least 2-Fold by Δ*luxS* Deletion

To investigate the role of LuxS in global gene expression, *E. coli* O157:H7-specific whole genome-scale transcriptome analysis was performed in both wild-type CI03J and its Δ*luxS* mutant strain ML03J. The results revealed 35 genes were differentially expressed by LuxS (Table 2). To verify our transcriptomic data, the six up- or downregulated genes in Table 2 were chosen and their transcription expression was confirmed using the real-time quantitative RT–PCR

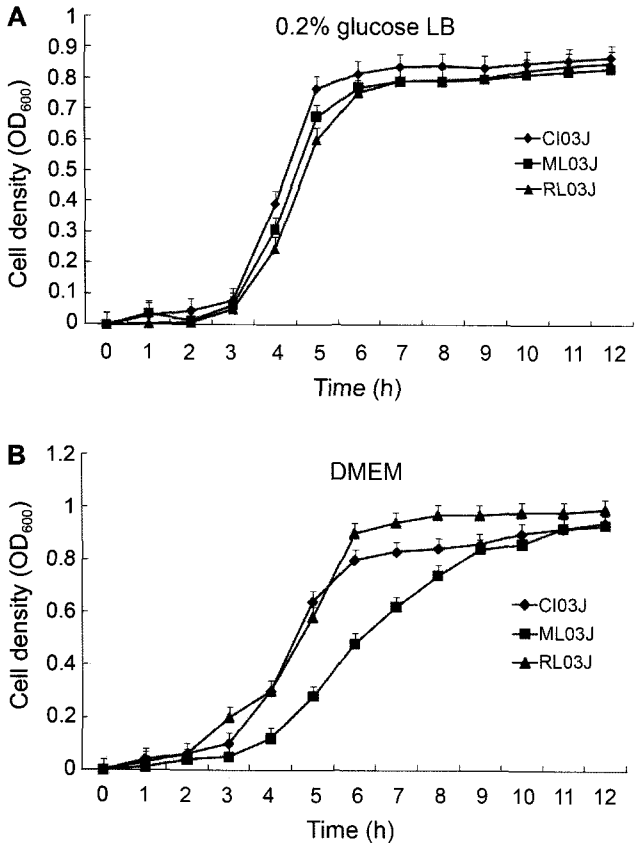

Fig. 2. Bacterial growth.
The wild-type (CI03J, filled diamond), Δ*luxS* mutant (ML03J, filled square), and its complemented (RL03J, filled triangle) strains were pre-cultured in LB containing 0.2% glucose (**A**) or DMEM (**B**) at 37°C with aeration. They were diluted to 1:1,000 with the same freshly prepared medium and their growth was monitored by measuring the OD_{600} under the same culture conditions. At least three independent experiments were performed and the data were represented as the average means ± SE.

(RT-qPCR) (Fig. 4). Consistent with the transcriptomic data in Table 2, we could observe transcriptional repression of the *fliC*, *cheY*, and *fliD* genes as well as transcriptional activation of the *clpB*, *asr*, and *ygaG* genes in the Δ*luxS* mutant strain, indicating that our transcriptomic data are highly reliable (Fig. 4). Among the 35 genes identified, 12 genes having functions mostly associated with motility or chemotaxis (*i.e.*, *flhD*; *fliA*, *C*, *D*, *S*, *Z*; *cheA*, *Y*, *and Z*) were upregulated, whereas the other 23 genes coding for chaperones or metabolic enzymes were downregulated (Table 2). However, there was no evident differential expression of any known virulence-associated genes such as LEE-induced genes that were previously defined as a part of the LuxS regulon. Interestingly, transcription expression of *gadAB*, whose gene products are responsible for the glutamate-dependent acid resistance [3, 13], was derepressed in ML03J (Table 2), implying a possible negative regulation of the *gadAB* genes by LuxS.

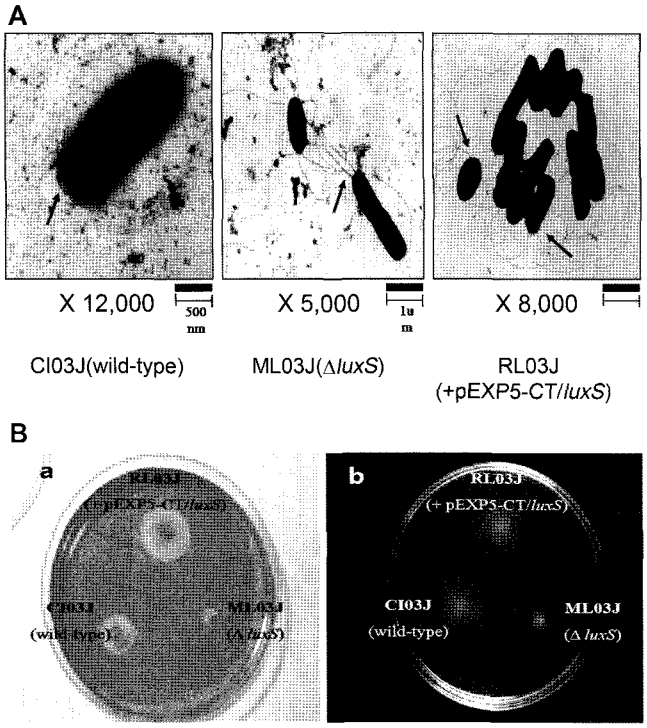

Fig. 3. Bacterial motility.
A. Microscopic analyses for direct visualization of intact flagella of the wild-type (CI03J), *luxS* mutant (ML03J), and its complemented (RL03J) strains by TEM. The arrows indicate the bacterial flagellum, and the magnitude of each picture is shown with the scale bars. **B.** Swarm motility of the wild-type (CI03J), *luxS* mutant (ML03J), and its complemented (RL03J) strains in either DMEM with 0.3% agar (panel a) or 0.3% tryptone agar (panel b).

Proteomic Analysis Reveals a Set of Cellular Proteins or ECPs Differentially Expressed by LuxS

We further analyzed the proteome profiles in CI03J and ML03J (Fig. 5). As summarized in Table 3, a total of 32 proteins were differentially expressed at least 2-fold by LuxS. Consistent with our transcriptome data and previous

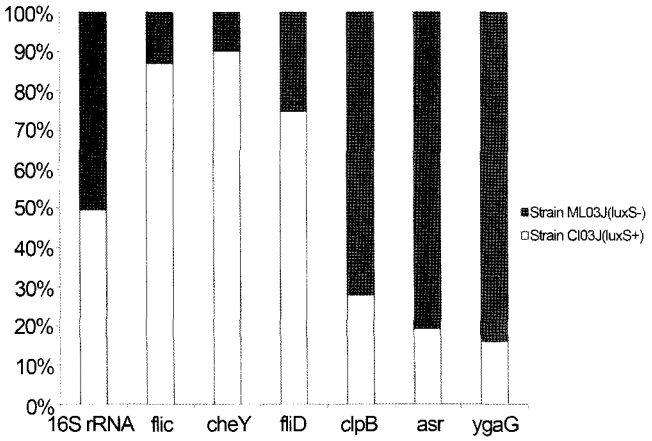

Fig. 4. RT-qPCR verification of the transcriptomic analysis.

Table 2. Transcriptome profiling of the wild-type *E. coli* O157:H7 and its isogenic *luxS* mutant strains.

Z number	Gene	Fold difference (CI03J vs. Δ*luxS*)	Description
Z3013	*fliC*	5.6	Flagellar biosynthesis; flagellin, filament structural protein
Z2936	*cheY*	2.9	Chemotaxis regulator transmits chemoreceptor signals to flagellar motor components
Z3014	*fliD*	2.8	Flagellar biosynthesis; filament capping protein; enables filament assembly
Z2935	*cheZ*	2.6	Chemotactic response; CheY protein phosphatase; antagonist of CheY as switch regulator
Z2940	*tar*	2.5	Methyl-accepting chemotaxis protein II, aspartate sensor receptor
Z2946	*flhD*	2.2	Regulator of flagellar biosynthesis, acting on Class 2 operons; transcriptional initiation factor
Z3012	*fliA*	2.2	Flagellar biosynthesis; alternative sigma factor 28; regulation of flagellar operons
Z3011	*fliZ*	2.1	Orf, hypothetical protein
Z3015	*fliS*	2.1	Flagellar biosynthesis; repressor of Class 3a and 3b operons (RflA activity)
Z2942	*cheA*	2.0	Sensory transducer kinase between chemo-signal receptors and CheB and CheY
Z4692	*rpsJ*	2.0	30S Ribosomal subunit protein S10
Z5711	*yjdA*	2.0	Putative vimentin
Z1391	*hyaC*	−2.0	Probable Ni/Fe-hydrogenase 1 b-type cytochrome subunit
Z2711	*ynhE*	−2.0	Orf, hypothetical protein
Z3526	*elaB*	−2.0	Orf, hypothetical protein
Z4424	*yqjI*	−2.0	Orf, hypothetical protein
Z4972	*yhjX*	−2.0	Putative resistance protein
Z5479	*hslV*	−2.0	Heat shock protein hslVU, proteasome-related peptidase subunit
Z5747	*mopB*	−2.0	GroES, 10 kDa chaperone binds to Hsp60 in pres. Mg-ATP, suppressing its ATPase activity
Z0606	*ybaS*	−2.1	Putative glutaminase
Z1418	*cbpA*	−2.1	Curved DNA-binding protein; functions closely related to DnaJ
Z2712	*ydiC*	−2.1	Orf, hypothetical protein
Z5678	*fdhF*	−2.1	Selenopolypeptide subunit of formate dehydrogenase H
Z5748	*mopA*	−2.1	GroEL, chaperone Hsp60, peptide-dependent ATPase, heat shock protein
Z4981	*cspA*	−2.2	Cold shock protein 7.4, transcriptional activator of hns
Z2215	*gadB*	−2.3	Glutamate decarboxylase isozyme
Z1390	*hyaB*	−2.4	Hydrogenase-1 large subunit
Z2769	*osmE*	−2.4	Activator of *ntrL* gene
Z4930	*gadA*	−2.5	Glutamate decarboxylase isozyme
Z0014	*dnaK*	−3.1	Chaperone Hsp70; DNA biosynthesis; autoregulated heat shock proteins
Z1389	*hyaA*	−3.2	Hydrogenase-1 small subunit
Z5351	*metE*	−3.4	Tetrahydropteroyltriglutamate methyltransferase
Z3886	*clpB*	−4.4	Heat shock protein
Z2591	*asr*	−6.9	Acid shock protein
Z3988	*ygaG*	−8.8	Orf, hypothetical protein

observations [2, 26, 28], we observed about 4.4-fold higher expression of flagellin (FliC) in the cellular fraction of the wild-type strain than in ML03J, implicating LuxS in the positive regulation of FliC (Table 3). Moreover, some metabolic enzymes such as aconitase B and biotin synthase were identified, whose expression was increased by LuxS. Surprisingly, we found a dramatic increase in expression of the pO157-encoded hemolysin in CI03J compared with ML03J, which has not been previously reported as a part of the LuxS regulon in *E. coli* O157:H7.

Although our transcriptome analysis could not detect any differential expression of previously known virulence genes, the secretome profiles showed increased expression of the LEE-encoded SepD (typeIII secreted protein) (about a 3.96-fold increase) as well as Shiga toxin 2B (Stx2B) subunit (about a 3.3-fold increase). Previously, it was demonstrated that a *luxS* mutation causes a minimal reduction in Stx expression *in vitro* [28], but no difference was observed *in vivo* [16]. Since Stx expression appeared to be increased by LuxS in our experimental conditions, we confirmed this result by comparing the cytotoxic effects on Vero cells by the culture supernatants from the wild-type and the Δ*luxS* mutant strains. As shown in Fig. 6, a significant reduction in Vero cytotoxicity was evident using the ML03J

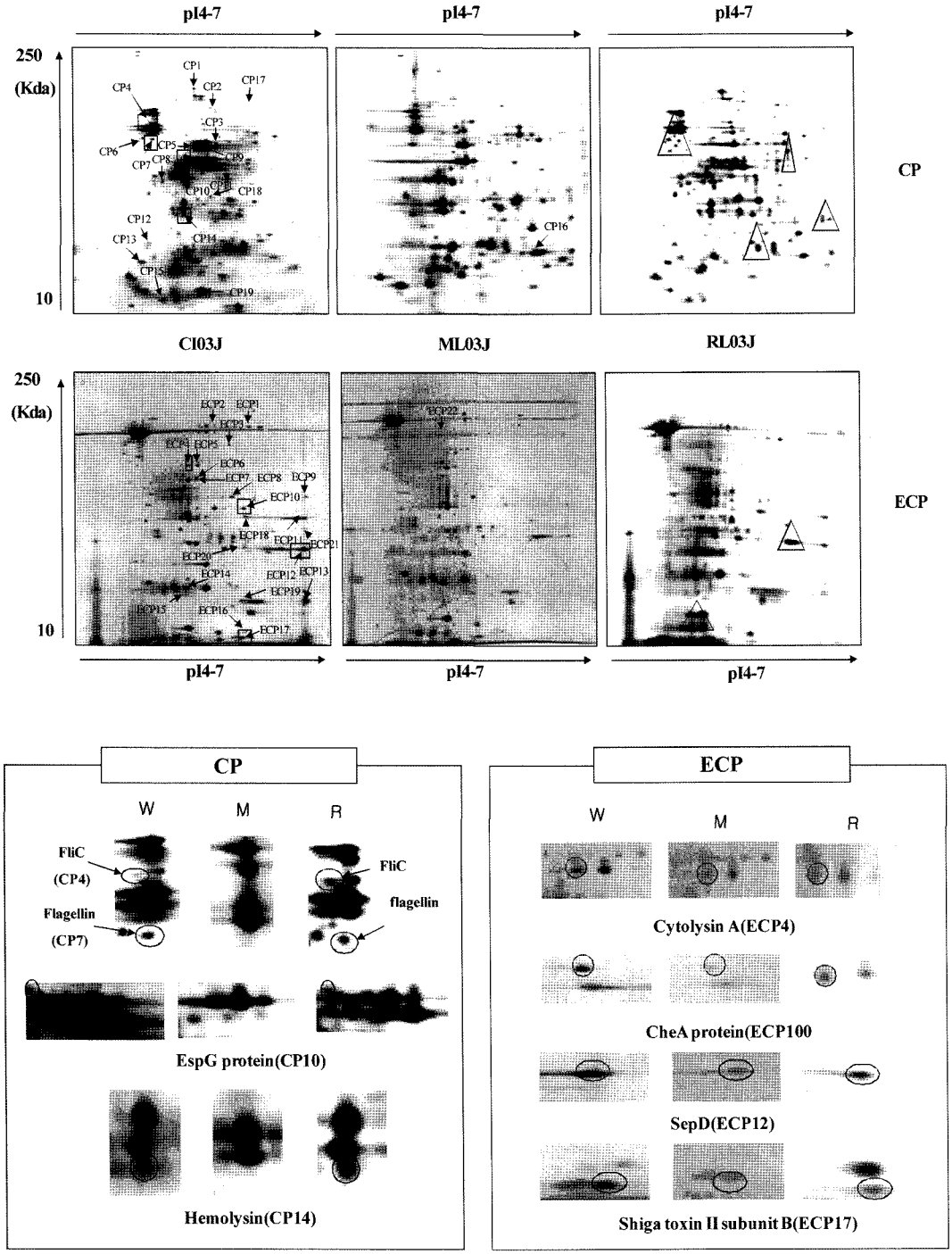

Fig. 5. Proteomic profiling.
Comparative two-dimensional gel electrophoresis was performed with either total cellular proteins (CP, the upper panel) or extracellular proteins (ECP, the middle panel) from the wild-type (CI03J), Δ*luxS* mutant (ML03J), and its complemented (RL03J) strains. Individual protein spots indicated by arrows were further identified by MALDI-TOF/MS (see Materials and Methods). Some representative spots identified are shown in the lower panel and all the identified proteins are listed in Table 3.

supernatant compared with supernatant of the wild type. In addition, we identified some LuxS-dependent proteins such as cytolysin A and phospholipase A, which have not been previously reported.

DISCUSSION

Presently, the *E. coli* O157:H7 Δ*luxS* mutant ML03J strain displayed no obvious growth or metabolic defects in 0.2%

Table 3. Proteomic analysis of the wild-type *E. coli* O157:H7 and its isogenic *luxS* mutant strains.

Spot ID	Protein function	Fold changes (CI03J vs. *luxS*)	MW (Da)	pI	Coverage (%)
Cellular proteins:					
CP01	Aconitase B	183	93,499	5.2	22.4
CP02	K+ efflux antiporter	14.6	67,796	5.7	19.8
CP04	FliC	4.4	68,134	4.5	19
CP03	Filament structural protein	153	51,295	4.5	28.5
CP04	Flagellin	187	57,809	4.7	26.7
CP05	EspG protein	2.1	43,901	5.2	8
CP06	Biotin synthase	4.1	39,648	5.3	10.7
CP07	Orf, hypothetical protein	2.2	26,423	4.7	13.4
CP08	Acyl-CoA thioesterase I	4.2	23,563	4.6	24.6
CP09	Hemolysin	166	33,555	5.2	12
CP10	Orf, hypothetical protein	2.5	17,911	4.9	26.8
CP11	Putative regulator	-2.4	24,678	6.5	13.7
CP12	Fcf1	2.5	35,595	5.6	16.8
CP13	Hemolysin-coregulated protein	3.0	19,256	5.5	37.2
Extracellular proteins:					
ECP01	Lipoprotein	3.4	42,860	5.9	77.1
ECP02	Aminopeptidase B	45	46,181	5.6	70.7
ECP03	Orf, unknown	2.1	39,574	5.6	25.4
ECP04	Cytolysin A	4.9	33,717	5.1	62
ECP05	HsdM protein	3.7	36,200	5.3	39.1
ECP06	Phospholipase A	2.9	33,171	5.2	67.8
ECP07	Putative regulator	3.3	24,268	5.7	21
ECP08	MbhA	8.3	23,780	6.9	65.4
ECP09	CheA	3.7	23,609	6.3	68.4
ECP10	EngB	3.9	23,561	6.9	92.4
ECP11	SepD	4.0	17,563	7	85.4
ECP12	Orf, hypothetical protein	-3.0	12,265	5.3	96.2
ECP13	Hydroxymethylbilane synthase	2.0	9,816	6.3	78.7
ECP14	Shiga toxin II subunit B	3.3	9,157	6.2	53
ECP15	Phenylacrylic acid decarboxylase-like protein	2.3	21,470	6.4	87.8
ECP16	Orf, hypothetical protein	2.1	19,536	6.1	78.7
ECP17	Acyl-CoA thioesterase I	2.5	23,622	6.9	76.4
ECP18	Orf, hypothetical protein	-6.1	37,276	5	94.2

glucose LB medium, could not utilize L-sorbose (although its biological significance is unknown in this microorganism), and produced less-functional flagella. Moreover, omics data revealed the involvement of LuxS in transcriptional activation of flagella/chemotaxis-related genes, repression of glutamate-dependent acid resistance genes, and increased expression of virulence factors such as Stx, hemolysin, and the LEE pathogenicity island. These results provide additional evidence for LuxS as a global regulator in *E. coli* O157:H7.

Previous studies demonstrated that *luxS* mutation in several bacterial species causes global changes in the expression of many genes that are involved in both biochemical and metabolic cellular processes [12, 15, 22, 25, 27, 28, 32, 34]. However, we observed little change in the ability of the Δ*luxS* mutant strain ML03J to utilize 49 carbohydrates and 24 amino acids compared with the wild-type strain CI03J. The sole difference between the wild-type and the Δ*luxS* mutant strains was the ability to degrade L-sorbose. Interestingly, CI03J could utilize L-sorbose as a single carbon source, but ML03J could not. Although the biological significance of L-sorbose utilization in the pathophysiology of *E. coli* O157:H7 is unclear, a recent study with some enteric bacterial pathogens such as pathogenic *E. coli* or *Shigella* suggested a possible link of bacterial ability to degrade L-sorbose with their pathomechanism(s) [21]. In addition, we speculate that LuxS-mediated QS signaling might be linked to the bacterial hunger response because ML03J did not show any obvious growth defects in nutrient-rich LB medium containing 0.2% glucose, but showed a slight growth reduction in DMEM.

It is well-known that the *luxS* gene product is responsible for production of the QS signaling molecules AI-2 and

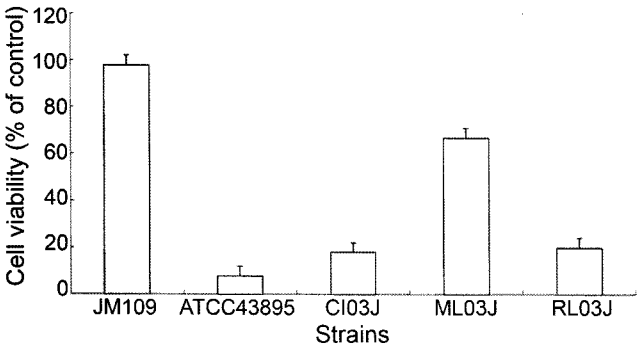

Fig. 6. Cytotoxicity of the culture supernants from the wild-type (CI03J), Δ*luxS* mutant (ML03J), and its complemented (RL03J) strains.

Vero cells were treated with bacterial supernatants and incubated for 1 h. Conversion of WST-1 to highly water-soluble formazan by viable cells was measured as described in Material and Methods. The data were obtained from three independent experiments and represented by average means ± SE in the percentage of cell viability. For all the assays, both *E. coli* O157:H7 ATCC43895 and *E. coli* JM109 strains were used as the positive and negative controls, respectively.

AI-3 [29, 31]. A recent study demonstrated that signaling mediated by AI-2 is transduced into cells through the QseBC two-component system, which controls biosynthesis of the flagellum in *E. coli* O157:H7 [7]. Surprisingly, our microscopic comparison of intact flagella in the wild-type and Δ*luxS* mutant strains revealed no visible differences between those strains, although our assessment was not quantitative. A previous report that the genes coding for flagella biosynthesis are partially (not completely) repressed by *luxS* mutation [28], combined with our swarm motility assay results demonstrating that ML03J is less motile than CI03J, prompts our hypothesis that the Δ*luxS* mutant strain ML03J might possess a functionally defective flagellum.

Our omics-based analysis identified a set of genes as a part of the LuxS regulon that were previously recognized (*i.e.*, several motility/chemotaxis or a few virulence genes such as *flhD*, *fliACDSZ*, *cheAYZ*, and *stx2B*) or unrecognized (*i.e.*, aconitase, *gadAB*, and hemolysin). Most interestingly, the glutamate-dependent acid resistance system (*gadAB*) [3, 13] seems to be downregulated by LuxS, whereas the pO157-encoded hemolysin is upregulated by LuxS. Although a previous study reported that QS signaling is involved in the production of hemolysin in *Staphylococcus aureus* [1], it is unclear whether or not the QS-dependent activation of the pO157-encoded hemolysin contributes to bacterial pathogenesis. In addition, we identified some LuxS-dependent proteins such as cytolysin A and phospholipase A, which have not been previously reported (to the best of our knowledge). It should be noted that we could not verify our proteomic data for all the identified proteins because of antibody availabilities. However, we repeated the 2D proteomic analysis three times independently and found that the expression patterns were very similar between those experimental trials (data not shown). Indeed, the *luxS*-dependent expression of some proteins such as Shiga toxin, flagellum, and the LEE-endoced SepD were confirmed by our functional assays (*i.e.*, Vero cytotoxicity of Shiga toxin and flagella motility) (Fig. 3 and 6) and other previous studies [18]. Further experiments will be needed to confirm and elucidate the roles of those genes in *E. coli* O157:H7, whose expressions were differentially regulated by LuxS.

Acknowledgments

This work was supported by a grant from the Pathogenic Proteome Management Program of the National Institute of Health, Ministry of Health, and Welfare, Republic of Korea (NIH 4800-4847-300) and also by the Second-Phase of the BK (Brain Korea 21) Project to JWY.

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