Biochemical and Biophysical Research Communications 436 (2013) 388–393

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Escherichia coli O157:H7 LPS O-side chains and pO157 are required for killing *Caenorhabditis elegans*

Min Youn [a], Kang-Mu Lee [a], So Hyun Kim [a], Jeesun Lim [a], Jang W. Yoon [b,*], Sungsu Park [a,*]

[a] Department of Chemistry and Nano Sciences (BK21), Ewha Womans University, Seoul 120-750, Republic of Korea
[b] Department of Food Science and Engineering, Ewha Womans University, Seoul 120-750, Republic of Korea

ARTICLE INFO

Article history:
Received 21 May 2013
Available online 6 June 2013

Keywords:
Escherichia coli O157:H7
Caenorhabditis elegans
Lipopolysaccharide
O-side chains

ABSTRACT

As a model host, the nematode *Caenorhabditis elegans* has been used for studying unknown pathogen–host interactions and identifying novel virulence factors in bacterial pathogens. Among the bacterial pathogens that can induce death of *C. elegans* is enterohemorrhagic *Escherichia coli* (EHEC) O157:H7, a major serotype of EHEC that causes hemorrhagic colitis and hemolytic uremic syndrome in humans and animals. However, it is unknown which EHEC O157:H7 factors are required for nematode death. In this study, bacterial ability to kill *C. elegans* was tested for several EHEC O157:H7 wild-type and mutant strains missing one virulence-associated factor, including Shiga toxins, enterohemolysin, pO157 (a large virulence plasmid in EHEC O157:H7), Type 3 secretion system, LuxS, and lipopolysaccharide (LPS) O-side chains. Our results demonstrate that only mutants lacking either pO157 or LPS O-side chains cause full attenuation in killing *C. elegans*. The LPS O-side chain-defective Δ*perA* mutant strain was not able to colonize in the intestine even at 24 h post-feeding with *C. elegans*, while the wild-type strain began to accumulate and colonize in the intestine as early as 3 h post-feeding. A simple complementation of the mutant strain with the plasmid carrying the intact *perA* gene *in trans* completely restored the production of LPS O-side chains, as well as the ability to kill *C. elegans*. Our results show that pO157 and PerA are required for EHEC O157:H7 to kill *C. elegans*.

1. Introduction

Escherichia coli O157:H7 is one of the most important serotypes of enterohemorrhagic *E. coli* (EHEC) that causes hemorrhagic colitis and hemolytic uremic syndrome in humans and animals [1,2]. The microorganism carries several established and putative virulence factors, including Shiga toxins (Stxs) [3], the locus of enterocyte effacement (LEE) pathogenicity island [4–7], and a large virulence plasmid (pO157) [1,8]. The ~92-kb virulence plasmid pO157 presents in almost all clinical isolates of EHEC O157:H7, but its biological significance is unclear [1]. The sequence of pO157 contains 100 open reading frames (ORFs), but only 19 have been characterized [8]. However, other not yet characterized virulence factors are believed to be required for full virulence of EHEC O157:H7 [2].

Several animals have served as models for EHEC O157:H7 infection to understand pathogen-host interactions [5,9,10]. Among the model systems, a bovine infection model is considered one of the most reliable and established model systems to study bacterial pathogenesis, especially bacterial carriage during infection, because cattle are known as a natural and principal reservoir of this microorganism [9–12]. However, this model system is quite expensive and requires a tightly-controlled and isolated facility.

The nematode *Caenorhabditis elegans* is a non-mammalian animal host that has been used to identify the virulence factors of various human pathogens [13–15]. The pathogen-mediated killing of *C. elegans* reveals two distinct killing phenotypes: fast-kill [14] and slow-kill [13,15]. The fast-kill phenotype is contact-independent and kills nematodes quickly within a few hours through diffusible toxin mediators [14], whereas the slow-kill phenotype is contact-dependent and kills nematodes slowly over several days through various infection processes such as bacterial colonization [13,15].

Recently, enteropathogenic *E. coli* (EPEC), which is a prototype of EHEC O157:H7, was found to kill *C. elegans* in either fast-kill [14] or slow-kill phenotypes [15]. In the slow-kill phenotype, EPEC can colonize, persist in the nematode intestine, and eventually kill *C. elegans*[15]. The model system has been also applied to EHEC O157:H7 infection to examine *in vivo* pathogenicity or evaluate the inhibitory effects of natural compounds on the virulence of EHEC O157:H7 [16]. Until now, however, little has been known about which EHEC O157:H7 factors are required for the slow killing of *C. elegans*.

* Corresponding authors. Fax: +82 2 871 7524 (J.W. Yoon), +82 2 3277 3419 (S. Park).
E-mail addresses: jwy706@gmail.com (J.W. Yoon), nanopark@ewha.ac.kr (S. Park).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.05.111

In this study, we examined the *C. elegans* killing ability of several EHEC O157:H7 wild-type and mutant strains missing one of the virulence-associated factors using a *C. elegans* killing assay [15]. The mutant strains carried a defined mutation in the previously established or putative virulence genes encoding Stxs (stx_1 or stx_2), T3SS-ATPase (*escN*) [6], enterohemolysin (*ehxA*) [17], pO157, LuxS (*luxS*) for quorum-sensing (QS) signaling [18], or GDP-*N*-acetyl-d-perosamine synthetase (*perA*), which is involved in the lipopolysaccharide (LPS) O-antigen biosynthesis of EHEC O157:H7 [9,19].

2. Materials and methods

2.1. Bacterial and nematode strains and growth media

The *E. coli* and *C. elegans* strains and plasmids in this study are listed in Table 1. All *E. coli* strains were maintained and grown at 37 °C in Luria–Bertani medium (LB; Difco). The germ line-defective and temperature-sensitive *C. elegans* strain SS104 was obtained from the *Caenorhabditis* Genetic Center at the University of Minnesota (Table 1), maintained at 15 °C on nematode growth media (NGM) (0.3% NaCl, 0.25% peptone, 5 mg/ml cholesterol, 1 M $CaCl_2$, 1 M $MgSO_4$, 1 M potassium phosphate buffer, 2.5% agar) plates, and propagated on the non-pathogenic feeder strain *E. coli* OP50 as previously described [15].

2.2. C. elegans killing assay

C. elegans killing assays were performed as previously described with some minor modifications [15]. The nematode *C. elegans* strain N2 was propagated on NGM plates at 15 °C and synchronized. The synchronized worms were fed on a lawn of the non-pathogenic feeder strain *E. coli* OP50 for 72 h until they reached Larva 4 (L4) stage, which is known to be hypersensitive to pathogens. The nematodes were moved onto NGM plates containing 10 μl of EHEC O157:H7 or *E. coli* OP50 strains pre-cultured in LB media at 37 °C overnight with shaking at 150 rpm and then incubated at 25 °C. Live worms were scored every 24 h. When worms did not respond to touch with a platinum wire pick, they were considered dead. At least three independent experiments were performed in triplicate, and the data were recorded as the mean TD_{50} value (time until 50% of nematodes were dead) ± standard deviation. To determine TD_{50} in a *C. elegans* killing assay, a curve was fitted to the data using SigmaPlot® version 11 (Systat Software, Inc.).

2.3. Fluorescence microscopy

Synchronized L4 nematodes were subjected to infection by EHEC O157:H7 or *E. coli* OP50 strains carrying pKEN-gfpmut2 (Table 1), a plasmid expressing green fluorescent protein (GFP), on NGM supplemented with 50 μg/ml of ampicillin (Amp) at 25 °C. After infection for 24 h, nematodes were removed using a platinum wire, placed in 500 μl of M9 media [20], and washed with the same media. Nematodes were transferred to glass microscope slides and visualized using a fluorescent microscope (Axiovert 100 M, Carl Zeiss, Jena, Germany).

2.4. LPS analysis

E. coli LPS was isolated from fresh cultures grown to an optical density at 600 nm (OD_{600}) of 1.0 in LB media at 37 °C by standard hot phenol extraction as previously described [21]. Isolated LPS was resolved on 12.5% polyacrylamide gel containing 0.5% sodium deoxycholate (DOC) as a denaturing agent instead of sodium dodecyl sulfate (SDS). DOC gels were pre-run for 5–10 min before loading LPS samples. LPS samples were boiled in buffers containing loading dye for 3 min prior to loading, run at 30 mA, and placed in 200 ml of fixing solution (40% ethanol, 5% acetic acid) overnight at 4 °C. The gels were visualized by silver staining as previously described by Tsai and Frasch [22].

2.5. Construction of EHEC O157:H7 ΔluxS mutant

Standard methods were utilized to conduct restriction digests, plasmid purification, PCR, ligation, transformation, and gel electrophoresis. To construct the *luxS*-disruption gene cassette, briefly, a sequence flanking the 5′ terminus of the *luxS* gene was amplified from the EHEC O157:H7 chromosome using the *luxS* LF (5′-AACATGCATGCCTCTGCCCGTATCTTAAGGTCTATG-3′) and *luxS* LR (5′-CGCGGATCCCTCTTTCTGGCATCACTTCTTT-3′) primers, and the 3′ flanking sequence was amplified using the *luxS* RF (5′-GCGCG GATCCATACCCTGGAGCACCTGTTTG-3′) and *luxS* RR (5′-AGCGC-GAGCTCGGATATTCCTCGTCTTGCTGG-3′) primers. The 5′ fragment was digested with *Sph*I and *Bam*HI, and the 3′ fragment was digested with *Bam*HI and *Sac*I. The pUC19 vector was digested with *Sph*I and

Table 1
Strains and plasmids used in the study.

Strain or plasmid	Description	Reference
E. coli strains		
OP50	Uracil auxotroph, a laboratory food source for *C. elegans*	
43895	*E. coli* O157:H7 (a human clinical isolate, stx_1^+/stx_2^+)	ATCC[a]
43894	*E. coli* O157:H7 (a human clinical isolate, stx_1^+/stx_2^+)	ATCC
85-170	*E. coli* O157:H7 (stx_1^-/stx_2^-)	ATCC
43895 Δ*ehxA*	ATCC 43895 carrying a deletion of enterohemolysin	[17]
43895 Δ*perA*	ATCC 43895 carrying a deletion of GDP-perosamine synthetase (lacking the LPS O-antigenic side chains)	[19]
43894 Δ*luxS*	ATCC 43894 carrying a deletion of LuxS (no AI-2 synthesis)	This study
43894 ΔpO157	ATCC 43894 lacking pO157 (originally referred as to 277)	J. Shaw
85-170 Δ*escN*	ATCC 43895 (*ΔescN::aphA-3*), T3SS-defective	J.W. Yoon
C. elegans strain		
SS104	*glp-4(bn2)I*	CGC[b]
Plasmids		
pKEN-gfpmut2	Plasmid expressing enhanced GFP, Amp^R	
pQE-30	IPTG-inducible protein expression vector	Qiagen
pQE-perA	Recombinant N-terminal 6xHis-PerA in pQE-30, Amp^R	This study

[a] American Type Culture Collection, Manassas, VA.
[b] *Caenorhabditis* Genetic Center, University of Minnesota, MN.

*Sac*I and ligated with the two digested *luxS* PCR products. The resultant plasmid was digested with *Bam*HI and ligated with the kanamycin (Km) resistance gene recovered from pUC4K digested with *Bam*HI. The suicide vector pCVD442 [23] containing the Amp-resistance gene was digested with *Sph*I and *Sac*I and ligated with the PCR product containing the Km-resistance gene digested with *Sph*I and *Sac*I. The ligation mixture was then transformed into EHEC O157:H7. Putative *luxS*-disrupted strains formed from the first and second crossover were detected by their Amp sensitivity and Km resistance. The Km-resistant colonies were again assessed via PCR and sequencing.

2.6. Inducible expression of a functional PerA

The structural gene of intact PerA in the chromosome of EHEC O157:H7 ATCC 43895 was amplified using gene-specific primers artificially incorporated with either *Sac*I or *Pst*I restriction sites, J23 (5′-AACGAGCTCAAATATATACCAGTTTACCA-3′) and J24 (5′-AACCTGCAGTGAATGACCTTTACAATATT-3′). The amplified products were purified, simultaneously digested with *Sac*I and *Pst*I (Invitrogen), and ligated into the IPTG (isopropyl β-D-thiogalactopyranoside)-inducible expression plasmid pQE-30 (Qiagen) after restriction by the same enzymes. The resultant plasmid pQE-perA was electroporated into the EHEC O157:H7 Δ*perA* mutant strain. Expression of the recombinant PerA tagged with the amino-terminal six histidine residues (6xHis-PerA) was induced by simple addition of 1 mM IPTG. A successful phenotypic restoration by this plasmid was confirmed by the absence or presence of a typical ladder-like LPS pattern in the EHEC O157:H7 Δ*perA* mutant and its complemented strains as described above.

2.7. Western blot analysis

Recombinant 6xHis-PerA expression was monitored by standard Western blot technique [20]. Briefly, total bacterial lysates grown with or without 1 mM IPTG were separated on 12% discontinuous SDS–polyacrylamide gels as described by Sambrook et al. [20]. Separated proteins were electrotransferred onto PVDF (polyvinylidene difluoride) membranes (Immobilon-P, Millipore). Blots were then blocked with 5% non-fat dried milk in PBS/Tween-20 (0.5%), incubated with anti-His antibody (1:5000 dilution) as recommended by the manufacturer (IG Therapy, Korea), reacted with horseradish peroxidase-conjugated anti-mouse IgG antibody (1:10,000 dilution) (Stressgen, U.S.A), and developed with a PicoEPD (Enhanced Peroxidase Detection) kit (ELPis-Bio, Korea) following the manufacturer's protocol.

2.8. Statistical analysis

Biological significance between the wild-type *E. coli* and mutant strains in killing of *C. elegans* was statistically analyzed by Student's *t*-test based on the results of at least three independent experiments.

3. Results

3.1. A slow-kill phenotype of EHEC O157:H7 in C. elegans

All the nematodes fed on *E. coli* OP50 looked normal during the initial periods of feeding (0–4 days post-feeding; data not shown) and displayed a reproducible survival kinetic over the experimental periods (Fig. 3B). In contrast, the nematodes fed with the three different wild-type EHEC O157:H7 strains (Table 1) showed aberrant and slowed movement within 1–2 days after feeding (data not shown) and began to die at 2–3 days post-feeding (Fig. 3B).

Table 2
TD_{50} and statistical analyses of *C. elegans* killing assays.

E. coli strain	TD_{50}[a]	*P* value[b] (*E. coli* O157:H7 WT)
OP50	206 ± 12.5	<0.05
O157:H7 43895 WT	130.3 ± 12.9	
O157:H7 43895 Δ*perA*	199.8 ± 4.6	<0.05
O157:H7 43985 Δ*perA* + pQE-30	186 ± 30.6	<0.05
O157:H7 43985 Δ*perA* + pQE-30-*perA*	115.5 ± 9.2	>0.05
O157:H7 43985 Δ*ehxA*	135.7 ± 10	>0.05
O157:H7 43894 WT	119.5 ± 61.9	
O157:H7 43894 ΔpO157	202.1 ± 18.3	<0.05
O157:H7 43984 Δ*luxS*	134.3 ± 15.5	>0.05
O157:H7 85-170 WT	136 ± 7.8	
O157:H7 85-170 Δ*escN*	150.1 ± 10.1	>0.05

[a] Mean TD_{50} values from at least three replicate assays using 12 nematodes each are presented as mean hours ± SD (standard deviation).
[b] Obtained by Student's *t*-test comparing TD_{50} values with those obtained for prototypical EHEC O157:H7 strains.

The mean TD_{50} values of the wild-type EHEC O157:H7 serotypes ATCC 43894, ATCC 43895, and 85–170 were 119.5, 130.3, and 136.0 h, respectively, which were significantly less than that of *E. coli* OP50 (mean TD_{50} of 206.0 h, $p < 0.05$; Table 2). These results imply that EHEC O157:H7 effectively kills *C. elegans* with a slow-kill phenotype [13,15].

3.2. Proliferation and accumulation of EHEC O157:H7 in the C. elegans intestine

Bacterial pathogens displaying a slow-kill phenotype are known to accumulate in the intestine of *C. elegans* [13,15]. Thus, we examined bacterial proliferation and accumulation in the *C. elegans* intestine. To visualize EHEC strains in the intestine, the GFP-expressing plasmid pKEN-gfpmut2 (Table 1) was transformed into both wild-type EHEC O157:H7 and non-pathogenic *E. coli* OP50 strains. Large amounts of GFP were detected in the nematode intestine fed wild-type EHEC O157:H7 ATCC 43894 (Fig. 1A) compared to those fed *E. coli* OP50 (Fig. 1C).

In agreement with our observation on green fluorescence (Fig. 1A), EHEC O157:H7 numbers in the nematodes rapidly increased and reached over 10^5 colony-forming units (CFU)/nematode within 3–6 h post-feeding (Fig. 2A). These results suggest that EHEC O157:H7 can proliferate and accumulate in the *C. elegans* intestine as early as 3 h post-feeding without killing *C. elegans*, which is similar to other bacterial pathogens such as *S.* Typhimurium that display a slow-kill phenotype [13].

3.3. pO157 and PerA are required for the killing of C. elegans

As shown in Table 2, significant attenuation in killing *C. elegans* by EHEC O157:H7 was observed only with the mutant strains lacking either the *perA* gene or pO157 ($p < 0.05$). The mean TD_{50} value of the ΔpO157 mutant strain was 202.1 h, which is much greater than that of the wild-type EHEC O157:H7 ATCC 43894 (mean TD_{50} of 119.5 h; Table 2). The ΔpO157 mutant strain was not able to persist or accumulate in the intestine of *C. elegans* at 24 h post-feeding (Fig. 2B; $p < 0.01$). These results imply that pO157 is required for EHEC O157:H7 to display a slow-kill phenotype in *C. elegans*. The mean TD_{50} value of the Δ*perA* mutant strain was 199.8 h, which is similar to that of the non-pathogenic feeder strain *E. coli* OP50 (mean TD_{50} of 206.0 h; Table 2), indicating that the Δ*perA* mutant strain became fully attenuated due to the loss of LPS O-side chains. This is supported by the observation that the Δ*perA* mutant strain could not accumulate in the intestine as well as the wild-type strain (Fig. 1B). To confirm whether or not EHEC O157:H7

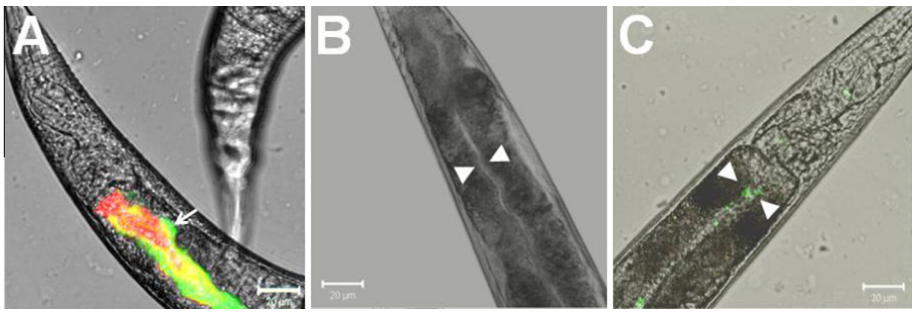

Fig. 1. Proliferation and accumulation of EHEC O157:H7 and its isogenic mutants in the *C. elegans* intestine. The images were obtained from *C .elegans* after feeding for 24 h on NGM agar at 25 °C with the following *E. coli* strains. (A) EHEC O157:H7 ATCC 43895. (B) *E. coli* OP50, negative control. (C) EHEC O157:H7 ATCC 43895 mutant strain carrying Δ*perA* (associated with biosynthesis of LPS O-side chains).

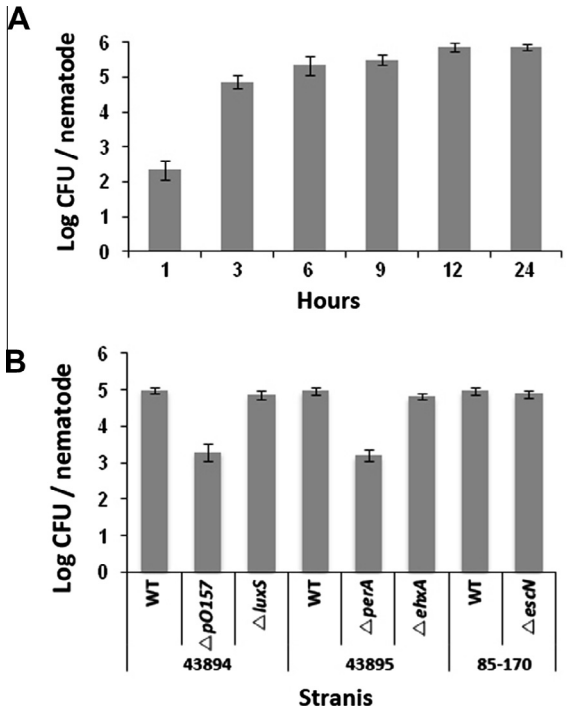

Fig. 2. Colonization of EHEC O157:H7 and its isogenic mutants in the nematode intestine. (A) Population of EHEC O157:H7 recovered from *C. elegans* incubated at 25 °C. (B) EHEC O157:H7 colonizes the nematode gut in greater numbers than mutants. Bleach-synchronized L4 nematodes were subjected to infection by EHEC strains on NGM agar plate. After 24 h of infection, three nematodes were harvested, washed to remove exterior bacteria, and supplemented with appropriate antibiotics. Washed worms were sonicated (Sonicate 450, Branson Ultrasonics, CT, USA) using a duty cycle of 25% for 25 s at 21 °C to rupture the cuticle of *C. elegans* and release ingested bacteria. Average CFUs per nematode exposed to EHEC O157 strains for 24 h and fed with heat-killed OP50 for 3 h are presented. Values represent the means of three replicate assays, and error bars indicate standard deviations.

LPS O-side chains are required for nematode killing *in vivo*, an IPTG-inducible expression plasmid pQE-perA was constructed and transformed into the Δ*perA* mutant strain (Table 1). A simple complementation of this mutant strain with pQE-perA carrying the intact *perA* gene was able to restore a typical ladder-like LPS pattern (Fig. 3A), as well as a characteristic slow-kill kinetic of *C. elegans* (Fig. 3B), compared to its isogenic Δ*perA* mutant strain carrying the plasmid vector pQE-30 (Fig. 3B). These results clearly demonstrate that the *perA* gene is required for EHEC O157:H7-mediated killing of *C. elegans*.

4. Discussion

We demonstrated that EHEC O157:H7 was able to infect, proliferate, and accumulate in the nematode intestine and effectively kill those worms in a slow-kill phenotype. Most importantly, it was found that the observed EHEC O157:H7-mediated killing of *C. elegans* requires pO157 and the intact LPS O-antigenic side chains (PerA).

Previous epidemiological studies suggest that almost all clinical isolates of EHEC O157:H7 carry a very stable, F-like virulence plasmid pO157 (1, 2, 8); however, the reason(s) why they all carry this plasmid has not been revealed, partly due to the absence of suitable animal models and the difficulty in curing the plasmid [1,2,8]. Although there have been conflicting results on the role of pO157 in adherence to epithelial cells [2], a recent study by Sheng et al. [9] showed that this plasmid can promote EHEC colonization in the gastro-intestinal tracts (GITs) using a bovine model. In agreement with this result, our *C. elegans* killing assays revealed that the EHEC O157:H7 ΔpO157 mutant was fully attenuated in nematode killing and not able to accumulate in the nematode intestine. Unfortunately, we cannot explain which pO157-encoded factors are responsible for the observed nematode killing. However, the isogenic Δ*ehxA* mutant strain did not show any attenuation in our *C. elegans* model system, implying that the *ehxA* gene in pO157 is not likely involved in the EHEC O157:H7-mediated killing of *C. elegans*. Our previous characterization of pO157 in EHEC O157:H7 demonstrated that this plasmid is required for survival through bovine GITs, persistence in farm environments, and maintenance of optimal membrane structure [24]. Since only a dozen of the pO157-encoded genes among the 100 ORFs have been functionally characterized [8], it would be interesting to determine which pO157-encoded factors are responsible for killing *C. elegans*.

As part of the outer membrane (OM) in Gram-negative bacteria, LPS is known to be an important molecule for maintaining OM structure and integrity, while interacting with host cells [25]. The molecule consists of three distinct structural components: the core oligosaccharide (OS) links a hydrophobic and endotoxic lipid A moiety to the long, antigenic *O*-polysaccharide side chains [25]. Interestingly, subtle modifications in both core OS and lipid A structures have been reported for EHEC O157:H7 LPS due to the differential expression of the genes for LPS biosynthesis by temperature [26,27]. Recently, the *perA* gene, originally referred to as an *rfbE* homologue [28], was characterized as a functional GDP-*N*-acetyl-d-perosamine synthetase [29,30]. A functional mutation of the *perA* gene in EHEC O157:H7 resulted in a lack of O-antigenic side chains in the OM LPS. Although its biological significance is not clearly understood, previous studies have demonstrated that the EHEC O157:H7 Δ*per* mutant strain shows a hyper-adherence phenotype accompanying characteristic AE lesions on cultured epithelial cells [28]. However, our data reveals that a functional

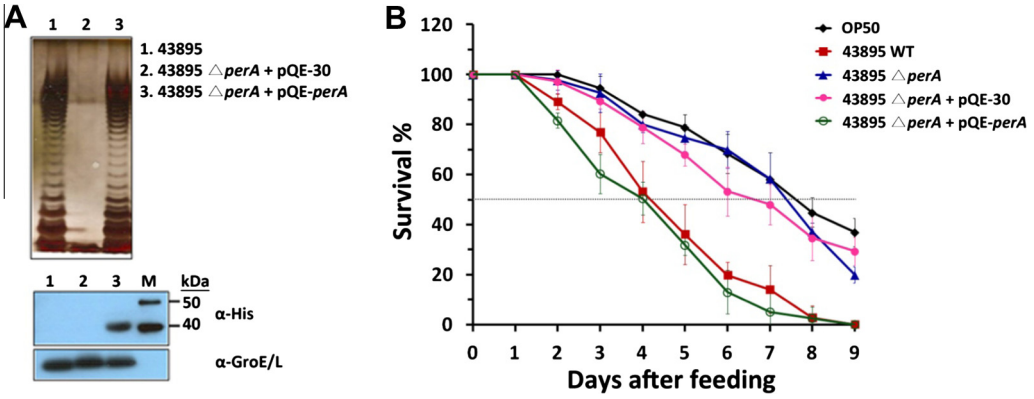

Fig. 3. Functional complementation of EHEC O157:H7 Δ*perA* mutant by PerA overexpression. An IPTG-inducible plasmid pQE-per carrying the intact *perA* gene was transformed into the EHEC O157:H7 Δ*per* mutant strain, and the resultant transformant was analyzed by *C. elegans* killing assays (see Section 2). (A) Bacterial LPS were isolated from the wild-type EHEC O157:H7 ATCC 43895, the Δ*perA* mutant, and its complemented strains and visualized by silver staining after 12.5% DOC-PAGE (upper panel). Overexpression of the recombinant 6xHis-tagged PerA was confirmed by Western blot analysis using the anti-His antibody (IG-therapy). As an internal control of the analysis, the anti-GroE/L antibody was used following the manufacturer's description (bottom panel). (B) The L4-stage nematodes were fed on NGM agar at 25 °C with the indicated *E. coli* strains. Surviving worms were counted over the period of infection. The data are presented as the average % of survival ± standard deviation from three independent experiments.

inactivation of the *perA* gene does not show such an anti-virulence phenotype in *C. elegans*. We cannot explain how the LPS O-side chains play a role in the EHEC O157:H7-mediated killing of *C. elegans*; however, we infer that LPS O-side chains may promote initial binding of EHEC O157:H7 to the intestinal epithelium of *C. elegans*. Supporting this notion, a recent study demonstrated that the same Δ*perA* mutant strain showed significantly reduced colonization and persistence at the bovine recto-anal junction mucosa, a principal colonization site of EHEC O157:H7 [12]. Although the mechanism behind the role of LPS O-side chains in bacterial colonization *in vivo*, our results support the idea that LPS O-antigenic side chains are a potential virulence determinant of EHEC O157:H7 during a host infection.

Recently, *C. elegans* has been applied to EPEC, which is a prototype of EHEC O157:H7 in evolution and shares some characteristics in the pathogenic mechanism such as the formation of the LEE-dependent AE lesions on the intestinal epithelium and the intimin-Tir interation [1]. Like EHEC O157:H7, EPEC is able to colonize and persist in the *C. elegans* intestine, produce intestinal AE lesions, and induce nematode death [14,15]. Interestingly, only the mutations in the genes encoding either tryptophanase [14] or Ler [15] showed strong or weak attenuation, respectively, in nematode killing by EPEC. However, no significant attenuation has been found with the EPEC mutants of the virulence-associated genes encoding Type IV bundle-forming pili, intimin, or Tir [5,30]. This suggests that other not yet characterized bacterial and/or host factors, such as EHEC/EPEC LPS O-side chains or *C. elegans* innate immunity, are likely more important for the EPEC- and/or EHEC-mediated killing of *C. elegans* than the previously defined virulence factors. It should be noted that EHEC O157:H7 expressing Stxs, one of the most potent cytotoxins, did not induce fast-killing of *C. elegans*, which is known to be typical for toxin-mediated killing of *C. elegans*. Since expression of such virulence factos is known to be quite sensitive to various environmental signals *in vivo* [7], we cannot rule out the possibility that the conditions (25 °C, NGM media) used for the nematode killing assays in this study may be not optimal for maximal expression of those EHEC virulence factors in nematode intestinal tracts.

In conclusion, our data imply that EHEC O157:H7 can kill the soil nematode *C. elegans* in a typical slow-kill kinetic, and both pO157 and intact LPS O-antigenic side chains are essential factors for the EHEC O157:H7-mediated killing of *C. elegans*.

Acknowledgments

This research was supported by the Public welfare & Safety research program (#2012M3A2A1051681) and the SRC program (#2012-0000647) through the National Research Foundation of Korea (NRF) funded by the Ministry of Future Creation and Science in Korea. J. W. Yoon was supported by RP-Grant 2010 of Ewha Womans University.

References

[1] J.B. Kaper, J.P. Nataro, H.L. Mobley, Pathogenic *Escherichia coli*, Nat. Rev. Microbiol. 2 (2004) 123–140.
[2] J.P. Nataro, J.B. Kaper, Diarrheagenic *Escherichia coli*, Clin. Microbiol. Rev. 11 (1998) 142–201.
[3] L. Johannes, W. Romer, Shiga toxins – from cell biology to biomedical applications, Nat. Rev. Microbiol. 8 (2010) 105–116.
[4] S. Tzipori, F. Gunzer, M.S. Donnenberg, L. De Montigny, J.B. Kaper, A. Donohue-Rolfe, The role of the *eaeA* gene in diarrhea and neurological complications in a gnotobiotic piglet model of enterohemorrhagic *Escherichia coli* infection, Infect. Immun. 63 (1995) 3621–3627.
[5] I. Vlisidou, F. Dziva, R.M. La Ragione, A. Best, J. Garmendia, P. Hawes, P. Monaghan, S.A. Cawthraw, G. Frankel, M.J. Woodward, M.P. Stevens, Role of intimin–tir interactions and the tir-cytoskeleton coupling protein in the colonization of calves and lambs by *Escherichia coli* O157:H7, Infect. Immun. 74 (2006) 758–764.
[6] A. Gauthier, J.L. Puente, B.B. Finlay, Enteropathogenic Escherichia coli's type III secretion system secretin requires components of the type III apparatus for assembly and localization, Infect. Immun. 71 (2003) 3310–3319.
[7] J.L. Mellies, A.M. Barron, A.M. Carmona, Enteropathogenic and enterohemorrhagic *Escherichia coli* virulence gene regulation, Infect. Immun. 75 (2007) 4199–4210.
[8] V. Burland, Y. Shao, N.T. Perna, G. Plunkett, H.J. Sofia, F.R. Blattner, The complete DNA sequence and analysis of the large virulence plamid of *Escherichia coli* O157:H7, Nucleic Acids Res. 26 (1998) 4196–4204.
[9] H. Sheng, J.Y. Lim, H.J. Knecht, J. Li, C.J. Hovde, Role of *Escherichia coli* O157:H7 virulence factors in colonization at the bovine terminal rectal mucosa, Infect. Immun. 74 (2006) 4685–4693.
[10] E.A. Dean-Nystrom, B.T. Bosworth, H.W. Moon, Pathogenesis of O157:H7 *Escherichia coli* infection in neonatal calves, Adv. Exp. Med. Biol. 412 (1997) 47–51.
[11] S.W. Naylor, J.C. Low, T.E. Besser, A. Mahajan, G.J. Gunn, M.C. Pearce, I.J. Mckendrick, D.G. Smith, D.L. Gally, Lymphoid follicle-dense mucosa at the terminal rectum is the principal site of colonization of enterohemorrhagic *Escherichia coli* O157:H7 in the bovine host, Infect. Immun. 71 (2003) 1505–1512.
[12] H. Sheng, J.Y. Lim, M.K. Watkins, S.A. Minnich, C.J. Hovde, Characterization of an *Escherichia coli* O157:H7 O-antigen deletion mutant and effect of the deletion on bacterial persistence in the mouse intestine and colonization at the bovine terminal rectal mucosa, Appl. Environ. Microbiol. 74 (2008) 5015–5022.

[13] A. Aballay, F.M. Ausubel, Programmed cell death mediated by *ced-3* and *ced-4* protects *Caenorhabditis elegans* from *Salmonella typhimurium*-mediated killing, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 2735–2739.
[14] A. Anyanful, J.M. Dolan-Livengood, T. Lewis, S. Sheth, M.N. Dezalia, M.A. Sherman, L.V. Kalman, G.M. Benian, D. Kalman, Paralysis and killing of *Caenorhabditis elegans* by enteropathogenic *Escherichia coli* requires the bacterial tryptophanase gene, Mol. Microbiol. 57 (2005) 988–1007.
[15] J.L. Mellies, A.M. Barron, K.R. Haack, A.S. Korson, D.A. Oldridge, The global regulator Ler is necessary for enteropathogenic *Escherichia coli* colonization of *Caenorhabditis elegans*, Infect. Immun. 74 (2006) 64–72.
[16] K.M. Lee, W.S. Kim, J. Lim, S. Nam, M. Youn, S.W. Nam, Y. Kim, S.H. Kim, W. Park, S. Park, Antipathogenic properties of green tea polyphenol epigallocatechin gallate at concentrations below the MIC against enterohemorrhagic *Escherichia coli* O157:H7, J. Food Protect. 72 (2009) 325–331.
[17] M.E. Bauer, R.A. Welch, Characterization of an RTX toxin from enterohemorrhagic *Escherichia coli* O157:H7, Infect. Immun. 64 (1996) 167–175.
[18] V. Sperandio, J.L. Mellies, W. Nguyen, S. Shin, J.B. Kaper, Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorrhagic and enteropathogenic *Escherichia coli*, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 15196–15201.
[19] J.Y. Lim, H. Sheng, K.S. Seo, Y.H. Park, C.J. Hovde, Characterization of an *Escherichia coli* O157:H7 plasmid O157 deletion mutant and its survival and persistence in cattle, Appl. Environ. Microbiol. 73 (2007) 2037–2047.
[20] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989.
[21] J. Guard-Petter, B. Lakshmi, R. Carlson, K. Ingram, Characterization of lipopolysaccharide heterogeneity in *Salmonella enteritidis* by an improved gel electrophoresis method, Appl. Environ. Microbiol. 61 (1995) 2845–2851.
[22] C.M. Tsai, C.E. Frasch, A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels, Anal. Biochem. 119 (1982) 115–119.
[23] N. Philippe, J.P. Alcaraz, E. Coursange, J. Geiselmann, D. Schneider, Improvement of pCVD442, a suicide plasmid for gene allele exchange in bacteria, Plasmid 51 (2004) 246–255.
[24] J.W. Yoon, J.Y. Lim, Y.H. Park, C.J. Hovde, Involvement of the *Escherichia coli* O157:H7 (pO157) ecf operon and lipid A myristoyl transferase activity in bacterial survival in the bovine gastrointestinal tract and bacterial persistence in farm water troughs, Infect. Immun. 73 (2005) 2367–2378.
[25] C.R. Raetz, C. Whitfield, Lipopolysaccharide endotoxins, Annu. Rev. Biochem. 71 (2002) 635–700.
[26] J.W. Yoon, S.A. Minnich, J.S. Ahn, Y.H. Park, A. Paszczynski, C.J. Hovde, Thermoregulation of the *Escherichia coli* O157:H7 pO157 *ecf* operon and lipid A myristoyl transferase activity involves intrinsically curved DNA, Mol. Microbiol. 51 (2004) 419–435.
[27] N.A. Kaniuk, E. Vinogradov, J. Li, M.A. Monteiro, C. Whitfield, Chromosomal and plasmid-encoded enzymes are required for assembly of the R3-type core oligosaccharide in the lipopolysaccharide of *Escherichia coli* O157:H7, J. Biol. Chem. 279 (2004) 31237–31250.
[28] S.S. Bilge, J.C. Vary, S.F. Dowell, P.I. Tarr, Role of the *Escherichia coli* O157:H7 O side chain in adherence and analysis of an *rfb* locus, Infect. Immun. 64 (1996) 4795–4801.
[29] C. Albermann, H. Beuttler, Identification of the GDP-*N*-acetyl-d-perosamine producing enzymes from *Escherichia coli* O157:H7, FEBS Lett. 582 (2008) 479–484.
[30] G. Zhao, J. Liu, X. Liu, M. Chen, H. Zhang, P.G. Wang, Cloning and characterization of GDP-perosamine synthetase (Per) from *Escherichia coli* O157:H7 and synthesis of GDP-perosamine *in vitro*, Biochem. Biophys. Res. Commun. 363 (2007) 525–530.