Lipopolysaccharide O-antigen of enterohemorrhagic Escherichia coli O157:H7 is required for killing both insects and mammals

Atsushi Miyashita1, Sunao Iyoda2, Kenichi Ishii1, Hiroshi Hamamoto1, Kazuhisa Sekimizu1 & Chikara Kaito1

1Laboratory of Microbiology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo, Japan; and 2Department of Bacteriology, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo, Japan

Correspondence: Chikara Kaito, Laboratory of Microbiology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 3-1, 7-chome, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. Tel.: +81 358 414 825; fax: +81 356 842 973; e-mail: kaito@mol.f.u-tokyo.ac.jp

Received 4 April 2012; accepted 14 May 2012.
Final version published online 11 June 2012.
DOI: 10.1111/j.1574-6968.2012.02599.x

Editor: Akio Nakane

Keywords
enterohemorrhagic Escherichia coli; silkworm model; lipopolysaccharide O-antigen; virulence.

Abstract
Studies of enterohemorrhagic Escherichia coli (EHEC) infection mechanisms using mammals require large numbers of animals and are both costly and associated with ethical problems. Here, we evaluated the pathogenic mechanisms of EHEC in the silkworm model. Injection of a clinically isolated EHEC O157:H7 Sakai into either the silkworm hemolymph or intraperitoneal fluid of mice killed the host animals. EHEC O157:H7 Sakai deletion mutants of the rfbE gene, which encodes perosamine synthetase, a monosaccharide synthetase of the O-antigen, or deletion mutants of the waaL gene, which encodes O-antigen ligase against the lipid A-core region of lipopolysaccharide (LPS), had attenuated killing ability in both silkworms and mice. Introduction of the rfbE gene or the waaL gene into the respective mutants restored the killing ability in silkworms. Growth of both mutants was inhibited by a major antimicrobial peptide in the silkworm hemolymph, moricin. The viability of both mutants was decreased in swine serum. The bactericidal effect of swine serum against both mutants was inactivated by heat treatment. These findings suggest that the LPS O-antigen of EHEC O157:H7 plays an important defensive role against antimicrobial factors in the host body fluid and is thus essential to the lethal effects of EHEC in animals.

Introduction
Infectious diseases caused by enterohemorrhagic Escherichia coli (EHEC) O157:H7 are a serious clinical problem and are associated with encephalopathy and nephropathy (Tarr, 1995; Law, 2000). An understanding of the molecular mechanisms of EHEC O157:H7 virulence is important for establishing effective therapeutic strategies. Unlike other E. coli strains, EHEC produces Shiga toxins and hemolysins. Shiga toxins are encoded by the stx1 and stx2 genes on the phage DNA that is integrated into the EHEC genome (Sato et al., 2003). The stx2 gene is required for EHEC to kill germ-free mice (Eaton et al., 2008). Hemolysins are encoded by elshCABD genes on the plasmid pO157 (Saitoh et al., 2008). These factors damage cultured intestinal epithelial cells (Obrig et al., 1988; Figueiredo et al., 2003). Bacterial motility and adherence to intestinal epithelial cells are considered to contribute to EHEC virulence (Levine et al., 1983; Holden & Gally, 2004). Expression of the flhDC gene, which encodes a transcription factor of flagellar genes, is activated when EHEC encounters nutrients (Tobe et al., 2011). EHEC attachment to intestinal epithelial cells forms attaching and effacing lesions. The locus of enterocyte effacement (LEE), a pathogenicity island of the EHEC genome, encodes many genes involved in the formation of attaching and effacing lesions. LEE contains the eae locus, which encodes a cell adhesive protein termed intimin (Jerse et al., 1991; Frankel et al., 1998). LEE also encodes the transcription factors Ler, GrlR, and GrlA, which regulate expression of the LEE genes (Elliott et al., 2000; Barba et al., 2005). Expression of the LEE genes is also regulated by PchA, PchB, PchC, and LrhA, which are encoded in other genome loci (Iyoda & Watanabe, 2004; Honda et al., 2009). LrhA not only activate the expression of LEE genes, but also activates the expression of the elshCABD, which encodes enterohemolysin and inactivates the expression of flagellar genes; thus, it is thought to
function as a switch to change the physiologic status of EHEC from a translocating phase to an adherence and toxin-producing phase (Lehnen et al., 2002; Honda et al., 2009; Iyoda et al., 2011). Although many EHEC O157:H7 genes are known to be involved in producing toxins, adherence and motility, it has not yet been investigated whether these factors, other than Shiga toxin 2, contribute to animal killing by EHEC.

EHEC O157:H7 possesses the O157 antigen on lipopolysaccharide (LPS). The LPS O-antigen in several Gram-negative bacteria, such as Shigella (West et al., 2005), Yersinia (Skurnik & Bengoechea, 2003), Salmonella (Ho et al., 2008), Burkholderia (Loutet et al., 2006), and Actinobacillus (Ramjeet et al., 2005), has a defensive role against host antimicrobial peptides. The LPS O-antigen of EHEC O157:H7 comprises N-acetyl-D-perosamine, L-fucose, D-glucose, and N-acetyl-D-galactose (Perry et al., 1986). N-acetyl-D-galactose is synthesized from galactose by GalE, GalT, GalK, and GalU (Genevaux et al., 1999). The galETKM deletion mutant of EHEC O157:H7, which has little O-antigen, has attenuated ability to colonize the infant rabbit intestine and is sensitive to antimicrobial polypeptides (Ho & Waldor, 2007). L-Fucose and N-acetyl-D-perosamine are monosaccharides specific for the LPS O-antigen (Wang & Reeves, 1998; Shimizu et al., 1999). Perosamine is found in the O-antigen of Vibrio cholerae O1, E. coli O157:H7, and Brucella spp. (Wu & Mackenzie, 1987; Samuel & Reeves, 2003). The rfb genes encode several synthases converting fructose-6-phosphate into GDP-α-perosamine, the structural unit of the O-antigen (Albermann & Piepersberg, 2001). In the last step of the biosynthesis reactions, the aminotransferase encoded by the rfbE gene synthesizes GDP-α-perosamine from GDP-4-keto-6-deoxymannosone (Albermann & Piepersberg, 2001). The rfbE (per) mutant of EHEC O157:H7 shows decreased viability in mouse and bovine intestine (Sheng et al., 2008). WecA protein polymerizes nucleotide-activated monosaccharides on the surface of the inner membrane of bacteria (Bengoechea et al., 2002), and WaAL protein ligates the polysaccharide to core-lipid A (Hug & Feldman, 2011). The waAL deletion mutant of uropathogenic E. coli has reduced viability in the mouse urinary tract (Billips et al., 2008). Based on these reports, the virulence properties of EHEC O157:H7 are thought to involve the LPS O-antigen, but whether the LPS O-antigen is required for animal killing by EHEC has not yet been determined.

To understand the molecular mechanisms of animal killing by pathogenic bacteria, bacterial virulence must be evaluated in animal models. A recent study revealed that oral administration of EHEC O157:H7 kills germ-free mice (Eaton et al., 2008; Fukuda et al., 2011). The use of germ-free mice for a genetic survey of EHEC virulence genes, however, would require very large numbers of animals and is thus associated with serious ethical and financial issues. Although insects lack an acquired immune system, they have innate immune systems that are highly conserved with mammals (Okada & Natori, 1983; Lehrer & Ganz, 2002). Antimicrobial peptides have a central role in the humoral innate immune system and are conserved among many living organisms, including insects and mammals (Okada & Natori, 1983; Meister et al., 1997; Natori et al., 1999; Natori, 2010). Similar to mammals, insects have a cytokine-like peptide that activates the expression of antimicrobial peptides (Meister et al., 1997; Tauszig et al., 2000; Ishii et al., 2010). Therefore, insects can be effectively used to investigate the molecular interactions between pathogenic bacteria and innate immune systems. Silkworms, larvae of the lepidopterans species Bombyx mori, are rarely killed by laboratory strains of E. coli, whereas they are killed by human pathogenic bacteria such as Staphylococcus aureus, V. cholerae, and Pseudomonas aeruginosa (Kaito et al., 2002). We identified S. aureus virulence genes using a silkworm infection model (Kaito et al., 2005, 2006; Matsumoto et al., 2007, 2010; Nagata et al., 2008; Ikuo et al., 2010; Miyazaki et al., 2011). Silkworms have several advantages as an infection model. They have a larger body than nematodes and fruit flies and can therefore be injected with quantitative amounts of bacterial solution for assessment of the bacterial virulence; that is, the 50% lethal dose (LD50) can be determined (Kaito & Sekimizu, 2007; Miyazaki et al., 2011). Moreover, infection experiments can be conducted at 37 °C, the human body temperature (Kaito et al., 2011). In this study, we utilized the silkworm as an animal model to investigate the molecular mechanisms of lethal infection by EHEC O157:H7.

**Materials and methods**

**Bacterial strains and culture conditions**

The bacterial strains and plasmids used in this study are listed in Supporting Information, Table S1. The E. coli strains were aerobically cultured in Luria–Bertani medium at 37 °C.

**Deletion of stx1, stx2, eae, flhDC, rfbE, and waAL genes in E. coli**

Deletions of E. coli genes were performed according to the ‘one-step inactivation method’ (Datsenko & Wanner, 2000). We designed primers having a complementary sequence to the upstream and downstream regions of the target genes and the kanamycin resistance gene of pKD4 (Table S2). Using these primers and pKD4 as a template,
DNA fragments were amplified by PCR and then electroporated into *E. coli* Sakai or SKI-5142. Gene deletion was confirmed by PCR. Deletion of the *waaL* gene was confirmed by Southern blot analysis.

**Silkworm infection experiment**

We purchased silkworm eggs (Fu/Yo × Tsukuba/Ne) from Ehime-Sanshu (Ehime, Japan). The hatched larvae were fed Silkmate (Nihon-Nosan Kogyo Co., Yokohama, Japan) at 27 °C. Fifth instar larvae were fed an antibiotic-free diet (Sysmex Corporation, Kobe, Japan) for 1 day and then injected with bacterial solution using a 1-mL syringe equipped with a 27-gauge needle. After injection, silkworms were incubated at 37 °C without food.

**Mouse infection experiment**

The study protocols were approved by the Animal Use Committee at the Graduate School of Pharmaceutical Science at the University of Tokyo. Jcl:ICR female mice (4 weeks old) were purchased from Clea Japan (Tokyo, Japan). The mice were intraperitoneally injected with *E. coli* cells suspended in phosphate-buffered saline (PBS) with 5% hog gastric mucin. Mice were kept in cages at 22 °C with autoclaved water and a gamma-ray-sterilized diet.

**Measurement of viable bacteria cells**

Samples were serially diluted with 0.9% NaCl solution and spread on at least two Luria–Bertani agar plates. The plates were incubated overnight at 37 °C, and the numbers of colonies that grew were counted.

**Preparation of a methanol extract from silkworm hemolymph**

Silkworm hemolymph was collected on ice and centrifuged at 3000 g for 5 min. The supernatant was thoroughly mixed with an equal volume of methanol and centrifuged at 3000 g for 5 min at 4 °C. The supernatant was dried using a rotary evaporator and dissolved in water. The amount of protein was determined by the Bradford method.

**Analysis of LPS**

LPS fractions were prepared according to the method of Coyne *et al.* (1994). The LPS fractions were mixed with a half volume of Laemml SDS sample buffer [150 mM Tris–HCl (pH 6.8), 6% SDS, 2% 2-mercaptoethanol, 30% glycerol, and 0.04% bromophenol blue], electrophoresed in 12.5% SDS–polyacrylamide gel, and transferred onto a polyvinylidene fluoride membrane (Immobilon-P; Millipore, Billerica, MA). The membrane was immunostained with rabbit polyclonal anti-O157 antibody (Denka Seiken, Tokyo, Japan).

**Growth inhibition by antimicrobial peptide**

Chemically synthesized moricin (Operon, Tokyo, Japan) was added to Luria–Bertani medium, and *E. coli* overnight cultures were added in 1 : 1000 dilution. The mixture was incubated at 27 °C for 5 h without shaking. After incubation, the number of viable cells was counted by plating the sample on Luria–Bertani agar plates.

**Measurement of bactericidal activity of swine serum**

*Escherichia coli* strains were cultured in Luria–Bertani medium to OD_{600} = 0.3. Bacterial cells were collected by centrifugation and suspended in PBS. Ten microliters of the bacterial suspension was mixed with fresh swine serum (NihonBiotest Co, Tokyo, Japan) and incubated at 37 °C for 90 min without shaking. After incubation, the number of viable cells was counted by plating the sample on Luria–Bertani agar plates.

**Results**

**Essential role of O-antigen in animal killing by EHEC O157:H7**

First, we compared the virulence of the EHEC O157:H7 Sakai strain and laboratory *E. coli* strain W3110 in silkworms. Injection of the Sakai strain into silkworm hemolymph and incubation at 37 °C for 20 h killed the silkworms (Fig. 1a). The LD_{50} of the Sakai strain was 4.3 × 10^{6} CFU per larva (Table 1). The LD_{50} of W3110 was 90 times higher than that of Sakai (Table 1).

Next, to identify the genes of EHEC O157:H7 required to kill silkworms, we investigated whether the supposed virulence factors of EHEC O157:H7 Sakai contribute to killing silkworms. The killing ability of double-deletion mutants of the *stx1* and *stx2* genes that encode Shiga toxin 1 and 2, respectively, in silkworms was indistinguishable from that of the parent strain, SKI5142 (Table 2). Moreover the deletion of *ehxA*, which encodes enterohemolysin, killed silkworms with an LD_{50} similar to that of the parent strain (Table 2). Similarly, the killing ability of the mutant with a deletion of *eae*, which encodes intimin and plays an essential role in bacterial adhesion to host cells, was indistinguishable from that of the parent strain (Table 2). Deletion of *flhDC*, which
We focused our attention on the LPS O-antigen of the outer membrane as a factor involved in the high virulence of EHEC O157:H7 against silkworms. We constructed a deletion mutant of the rfbE gene in the Sakai background, which encodes perosamine synthase, a monosaccharide component of the O-antigen that is specific for O157:H7. We also constructed a deletion mutant of the waaL gene that encodes a ligase of the O-antigen to core-lipid A (Fig. S1a and b). To confirm the absence of the LPS O-antigen in these mutants, we immunostained LPS fractions of these mutants using anti-O157 immunoglobulin. The findings indicated that both deletion mutants, rfbE and waaL, lacked the LPS O-antigen (Fig. S1c). We further confirmed that introducing rfbE or waaL into the respective mutant restored the LPS O-antigen (Fig. S1c). Thus, the rfbE and waaL genes are responsible for the LPS O-antigen in O157:H7 Sakai. Next, we examined whether rfbE and waaL deletion mutants had decreased virulence against silkworms. The LD₅₀ values of the rfbE and waaL mutants against silkworms were 1.4 × 10⁸ CFU per larvae and 2.1 × 10⁵ CFU per larvae, respectively, 30-fold higher than the LD₅₀ of the Sakai strain (Fig. 1a and b, Table 1). Furthermore, introduction of rfbE and waaL into the respective mutant decreased the LD₅₀ values in silkworms (Fig. 1a and b, and Table 1). These findings suggest that the rfbE and waaL genes are required for the silkworm-killing ability of EHEC O157:H7. In other words, the LPS O-antigen has an essential role in silkworm lethality because of EHEC O157:H7.

We then examined the virulence of EHEC O157:H7 in mice. Intraperitoneal injection of the Sakai strain killed mice, whereas the rfbE and waaL mutants had attenuated

<table>
<thead>
<tr>
<th>Strain</th>
<th>LD₅₀ (N = 5)</th>
<th>LD₅₀ ratio</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sakai (WT)</td>
<td>4.3 ± 1.1 10⁶</td>
<td>3 1</td>
<td></td>
</tr>
<tr>
<td>SKI-5440/pMW118 (ΔrfbE)</td>
<td>1.4 ± 0.44 10⁸</td>
<td>3 33</td>
<td>0.0382 (vs. WT)</td>
</tr>
<tr>
<td>SKI-5440/pRFbE (rfbE⁺)</td>
<td>8.8 ± 0.31 10⁸</td>
<td>3 2</td>
<td>0.0422 (vs. ΔrfbE)</td>
</tr>
<tr>
<td>SKI-5441/pMW118 (ΔwaaL)</td>
<td>2.1 ± 0.20 10⁸</td>
<td>2 49</td>
<td>0.0008 (vs. WT)</td>
</tr>
<tr>
<td>SKI-5441/pMW118 (waaL⁺)</td>
<td>2.8 ± 0.50 10⁷</td>
<td>2 7</td>
<td>0.0126 (vs. ΔwaaL)</td>
</tr>
<tr>
<td>W3110</td>
<td>4.1 ± 0.05 10⁸</td>
<td>2 94</td>
<td>&lt; 0.0001 (vs. WT)</td>
</tr>
</tbody>
</table>

Twofold serial dilutions of *Escherichia coli* overnight cultures were injected into silkworms (n = 5), and the surviving silkworms were counted at 20 h after the injection. The LD₅₀ value was determined by logistic regression. Data represent mean ± standard error. Student t-test P-values are presented. N, number of experiments; LD₅₀ ratio, the ratio of the LD₅₀ against that of Sakai (WT).
requirement of LPS O-antigen for EHEC virulence

Table 2. Killing ability of virulence gene deletion mutants of EHEC O157:H7 against silkworms

<table>
<thead>
<tr>
<th>Strain</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt; (CFU)</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt; ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SKI-5142 (parent)</td>
<td>6.9 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>SKI-5500 (Δstx1/stx2)</td>
<td>8.6 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1.2</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SKI-5142 (parent)</td>
<td>8.8 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>SKI-5171 (ΔehxA)</td>
<td>8.3 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.9</td>
</tr>
<tr>
<td>Experiment 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SKI-5142 (parent)</td>
<td>4.8 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>SKI-5510 (Δeae)</td>
<td>6.5 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.4</td>
</tr>
<tr>
<td>SKI-5301 (ΔftrhA)</td>
<td>3.0 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.6</td>
</tr>
<tr>
<td>Experiment 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SKI-5142 (parent)</td>
<td>8.4 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>SKI-5201 (ΔfthDC)</td>
<td>9.5 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Twofold serially diluted overnight cultures of Escherichia coli strains were injected into silkworms (n = 2–8) and the surviving silkworms were counted at 20 h after the injection. The LD<sub>50</sub> values were determined by logistic regression. LD<sub>50</sub> ratio, the ratio of the LD<sub>50</sub> against that of SKI-5142 (parent) in each experiment.

Table 3. Killing ability of the rfbE and waaL deletion mutants of EHEC O157:H7 against mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt; (CFU)</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt; ratio</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sakai (WT)</td>
<td>&lt;1.0 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>SKI-5440/pMW118 (ΔrfbE)</td>
<td>1.0 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>&gt;10</td>
<td>ND</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sakai (WT)</td>
<td>1.1 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>0.0115</td>
<td></td>
</tr>
<tr>
<td>SKI-5440/pMW118 (ΔrfbE)</td>
<td>1.1 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>10</td>
<td>0.0450</td>
</tr>
</tbody>
</table>

Twofold serial dilutions of Escherichia coli cells were intraperitoneally injected into ICR mice (n = 5), and surviving mice were counted at 18 h after the injection. The LD<sub>50</sub> was determined by logistic regression. Likelihood ratio test P-values between Sakai (WT) and the gene deletion mutant are shown.

Requirement of the O-antigen for bacterial growth in silkworm hemolymph

We hypothesized that the attenuated killing ability of LPS O-antigen-deficient rfbE mutant was because of its growth deficiency in silkworms. The number of viable cells of the Sakai strain increased in the silkworm hemolymph from 1.5 to 6 h after the injection, whereas that of the rfbE mutant decreased from 0.5 to 6 h (Fig. 2a). Invertebrate animals, including silkworms, do not possess antibodies, and the innate immune system defends them from bacterial infection. Therefore, we considered that the LPS O-antigen in EHEC O157:H7 is necessary for defense against the silkworm innate immune responses. Innate immune responses exclude foreign substances such as bacteria via phagocytosis by hemocytes (blood cells) or bactericidal action of humoral factors, including antimicrobial peptides. Silkworm hemocytes incorporated a comparable number of Sakai cells and rfbE mutant cells in vitro (data not shown). We then examined whether the rfbE mutant had increased sensitivity against the silkworm humoral factors. We cultured the Sakai strain and the rfbE mutant in liquid medium supplemented with silkworm hemolymph supernatant for 5 h and measured the number of viable cells. The hemolymph supernatant decreased the number of viable cells of the rfbE mutant in a dose-dependent manner, but had no effect on the number of viable cells of the Sakai strain (Fig. 2b). Therefore, we assumed that the LPS O-antigen of EHEC O157:H7 is required for resistance against silkworm humoral antimicrobial factors. The antimicrobial activity of silkworm hemolymph was not inactivated by heat treatment of the supernatant fraction at 100 °C for 15 min (data not shown). In addition, this activity was recovered after methanol extraction (data not shown). These characteristics are consistent with those of antimicrobial peptides of insect hemolymph; thus, we assumed that the antimicrobial activity of the silkworm hemolymph is because of the antimicrobial peptides. The antimicrobial peptides of insects are induced by exposure to bacteria (Furukawa et al., 1999). To verify whether the antimicrobial activity of the silkworm hemolymph supernatant is caused by the antimicrobial peptides, we examined whether injection of Sakai cells into silkworms induced the antimicrobial activity. We injected saline or Sakai cell suspension into silkworms and prepared a methanol extract from the hemolymph 8 h after the injection. The methanol extract of the hemolymph from silkworms injected with the Sakai strain more effectively inhibited rfbE mutant growth than that from silkworms injected with saline (Fig. 2c). The growth inhibitory activity of silkworm hemolymph was also induced by injecting rfbE mutant cells into silkworms (data not shown). These results suggest that antimicrobial peptides are responsible for the growth inhibitory activity of silkworm hemolymph against the rfbE mutant.

Moricin is a major antimicrobial peptide produced in the silkworm hemolymph (Hara & Yamakawa, 1995). We examined whether the rfbE and waaL mutants showed increased sensitivity to moricin. We cultured these mutants in liquid medium supplemented with moricin...
and measured the number of viable cells. The numbers of viable cells of the rfbE and waaL mutants were smaller than that of the parent strain (Fig. 3a). The decreased numbers of viable cells of the rfbE and waaL mutants were restored by introducing the intact rfbE and waaL genes, respectively, into each mutant (Fig. 3a). In the absence of moricin, the growth of the two mutants was comparable to that of the parent strain (Fig. 3a). These findings suggest that the LPS O-antigen contributes to the resistance of EHEC O157:H7 to moricin.

**Requirement of the LPS O-antigen for EHEC O157:H7 survival of in mammalian serum**

We next examined whether the LPS O-antigen of EHEC O157:H7 contributes to resistance against mammalian humoral innate immune factors. The number of viable cells of the rfbE and waaL mutants was decreased to less than one-hundredth that of the parent strain in swine serum (Fig. 3b). The decreased cell number of the rfbE and waaL mutants in swine serum was restored by introducing the intact rfbE and waaL genes, respectively (Fig. 3b). In the absence of swine serum, the cell numbers of these mutants were comparable with that of the parent strain (Fig. 3b). Heat treatment, which is widely used for the inactivation of complements, abolished the bactericidal activity of swine serum against the rfbE and waaL mutants (Fig. 3b). Therefore, these findings suggest that the LPS O-antigen of EHEC O157:H7 is required for resistance against the heat-susceptible antimicrobial factors of swine serum.

**Discussion**

The findings of the present study indicate that EHEC O157:H7 kills silkworms, and the LPS O-antigen of this pathogen is required for this silkworm-killing effect. In addition, the LPS O-antigen-dependent killing ability of EHEC O157:H7 was also observed in a mouse intraperitoneal infection model. Shiga toxin 2 was not required for EHEC O157:H7 to kill silkworms (Table 1). Other researchers have reported that Shiga toxin 2 is required for EHEC O157:H7 to kill germ-free mice (Eaton et al., 2008). These results indicate that EHEC O157:H7 harbors virulence factors required for killing both insects and mammals as well as factors required only for killing mammals. Thus, the silkworm infection model is effective for evaluating the animal killing ability of EHEC O157:H7 and is useful for identifying the essential factors, including the LPS O-antigen, of EHEC O157:H7 that are required to kill animals.

We also demonstrated that the O-antigen-deficient mutant of EHEC O157:H7 could not grow in silkworm hemolymph, whereas the parent strain could grow. The growth inhibitory factor of the silkworm hemolymph against the O-antigen-deficient mutant may be an antimi-
crobial peptide, because the factor(s) is heat resistant and methanol soluble. In addition, the O-antigen-deficient mutant was sensitive to the antimicrobial peptide, moricin (Fig. 3a). These results suggest that the LPS O-antigen of EHEC O157:H7 is required for resistance against antimicrobial peptides, which allows for bacterial growth in the silkworm hemolymph and the subsequent killing of silkworms. This concept is further supported by previous reports that the LPS O-antigen contributes to the defense against antimicrobial peptides in several Gram-negative bacteria (Skurnik & Bengoechea, 2003; Ramjeet et al., 2005; West et al., 2005; Loutet et al., 2006; Ho et al., 2008). Furthermore, the O-antigen-deficient mutants of EHEC O157:H7 were sensitive to heat-susceptible antimicrobial factors of serum. Because the major heat-susceptible antimicrobial factor of serum is a complement factor, we considered that the LPS O-antigen of EHEC O157:H7 is required for resistance against complement activity. We assume that these two functions of the LPS O-antigen cooperatively contribute to the ability of EHEC O157:H7 to kill animals.

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research. This study was supported in part by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) and the Genome Pharmaceutical Institute.

References


Fig. 3. The rfbE and waaL mutants of EHEC O157:H7 Sakai are sensitive against animal antimicrobial factors. (a) Bacterial cells (1–2 × 10^5 CFU) of Sakai (WT), SKI-5440 (ΔrfbE) transformed with a vector plasmid (pMW118) and a plasmid carrying the intact rfbE gene (prfbE), and SKI-5441 (ΔwaaL) transformed with a vector plasmid (pMW118) and a plasmid carrying the intact waaL gene (pwaaL) were cultured in liquid medium supplemented with 100 µg ml^−1 moricin at 27 °C for 5 h, and the number of viable cells was measured. Data represent the mean ± standard error from two independent experiments. *Student’s t-test P-value < 0.05. (b) Bacterial cells (1 × 10^5–10^6 CFU) of Sakai (WT), SKI-5440 (ΔrfbE) transformed with a vector plasmid (pMW118) and a plasmid carrying the intact rfbE gene (prfbE), and SKI-5441 (ΔwaaL) transformed with a vector plasmid (pMW118) and a plasmid carrying the intact waaL gene (pwaaL) were incubated in PBS supplemented with swine serum for 90 min at 37 °C, and the number of viable cells was measured. Data represent the mean ± standard error of two independent experiments. *Student’s t-test P-value < 0.05.


Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Construction of the O-antigen deficient mutants of EHEC O157:H7 Sakai.

**Table S1.** A list of bacterial strains and plasmids used.

**Table S2.** Primers used in this study.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.