Urease activity of enterohaemorrhagic Escherichia coli depends on a specific one-base substitution in \textit{ureD}

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The authors previously reported that most enterohaemorrhagic 	extit{Escherichia coli} (EHEC) strains do not express urease activity, despite having the urease gene. This study compared the nucleotide sequences of the urease gene clusters of a urease-activity-positive and a urease-activity-negative strain. The results showed that in the urease-activity-negative strain, \textit{ureD}, a gene encoding a chaperone protein, had a single base substitution that encoded a premature stop codon resulting in a short ORF. The premature stop codon in \textit{ureD} was commonly found in urease-activity-negative EHEC strains, but not in urease-activity-positive strains. Urease activity was detected after complementing the urease-activity-negative strain with \textit{ureD} from the urease-activity-positive strain. Furthermore, introduction of the urease gene cluster from the urease-activity-negative strain into an amber suppressor phenotype 	extit{Escherichia coli} strain, DH5\textit{x}, conferred the ability to produce the active urease. These results suggest that the lack of urease activity in most EHEC strains is due to a premature stop codon in \textit{ureD}.

INTRODUCTION

Enterohaemorrhagic 	extit{Escherichia coli} (EHEC) is a foodborne pathogen that can cause severe clinical manifestations such as haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) (Verweyen et al., 2000). Although there are a number of different serotypes of EHEC strains, serotype O157:H7 strains are more commonly isolated in the clinical context than other serotypes (Griffin & Tauxe, 1995; Makino et al., 1992). Other possible virulence determinants on the chromosome and on virulence plasmid pO157 are also known. However, the roles of those genes in the pathogenesis of EHEC remain unclear (McDaniel & Kaper, 1997; Gyles et al., 1998; Makino et al., 1998; Perna et al., 1998).

The genome sequences of two EHEC strains isolated from patients in the USA and Japan have been reported (Hayashi et al., 2001; Perna et al., 2001). The sequences revealed that the urease gene cluster is present on the chromosome of both strains. Urease genes from both strains are identical in genetic organization. The urease genes are composed of three structural genes, \textit{ureA}, \textit{ureB} and \textit{ureC}, and four accessory genes, \textit{ureD}, \textit{ureE}, \textit{ureF} and \textit{ureG} (Hayashi et al., 2001; Perna et al., 2001). Based on the genome sequence, the urease genes are most similar to those of 	extit{Klebsiella aerogenes} (Mulrooney & Hausinger, 1990; Lee et al., 1992).

Recently, we reported that, among pathogenic 	extit{E. coli} strains, the urease gene is specifically associated with EHEC strains (Nakano et al., 2001). This finding suggests that the urease gene could be a useful genetic marker for the detection of EHEC strains. In that study, we also demonstrated that most EHEC strains showed no urease activity, despite the presence of the urease gene. Although other studies have shown that urease genes are regulated by environmental signals in various species (Mobley et al., 1995), the regulation of the urease genes and the urease activity of EHEC remain unclear. Recently, Heimer et al. (2002) demonstrated that the urease genes of EHEC could be regulated both by Fur and by an unidentified trans-acting factor. However, it is not known why most EHEC strains do not express urease activity.

Here, we analysed the nucleotide sequence of the entire urease gene cluster derived from a urease-activity-positive EHEC strain and compared it with that of the urease-activity-negative EHEC O157:H7 strain RIMD0509952, the genome sequence of which has been reported (Hayashi et al., 2001; Watanabe et al., 1996). We demonstrate that the exhibition of urease activity by EHEC strains depends on a specific one-base substitution in the \textit{ureD} gene.

Abbreviations: EHEC, enterohaemorrhagic 	extit{Escherichia coli}; HC, haemorrhagic colitis; HUS, haemolytic uraemic syndrome.
METHODS

Bacterial strains and plasmids. EHEC O157:H7 strain RIMD0509952 (referred to as O157 Sakai) (Hayashi et al., 2001; Watanabe et al., 1996) was used as the standard strain in this study. EHEC Out:H+ strain RIMD05091612 (referred to as the Ure-1 strain), isolated from cattle feces, was used as the standard for a urease-activity-positive strain. A total of 158 EHEC strains isolated from various sources (68 from humans, 71 from cattle, 11 from deer, 6 from sheep and 2 from seagull) (Makino et al., 2003) were used in this study to examine the size of the entire urease gene cluster and analyse the nucleotide sequence between ureD and ureA. Other bacterial strains and plasmids used in this study are listed in Table 1. Bacterial strains were generally grown in Luria–Bertani (LB) broth or M9 medium with 1 μM NiCl2 (Heimer et al., 2002).

Genetic manipulations. Genomic DNA from E. coli was extracted by the usual method (Wilson, 1994). Plasmid DNA used for sub-cloning or nucleotide sequence analysis was extracted from E. coli DH5α carrying recombinant plasmids using the QIAprep Spin Plasmid Kit (Qiagen) according to the manufacturer’s recommendations. Other procedures for DNA manipulations, such as cloning of DNA fragments, restriction endonuclease treatment, DNA ligation and transformation, were carried out using standard protocols (Sambrook et al., 1989). All restriction enzymes were purchased from Toyobo and the DNA ligation kit was from Takara Bio.

Oligonucleotide primers and PCR conditions. The sequences of oligonucleotide primers used in this study are described in Table 2. The oligonucleotide primers were constructed from the O157 Sakai genome sequence (Hayashi et al., 2001). Standard PCR conditions were as follows: after 3 min denaturation, a cycle of denaturation at 94°C for 30 s, annealing at appropriate temperature for 30 s and extension at 72°C for 1 min was repeated 30 times; PCR was performed using Gene Taq DNA polymerase (Wako Pure Chemical Industries) and Takara Ex Taq (Takara Bio). PCR conditions for determining the length of the urease gene cluster using primers LA1 and LA2 were as follows: after 3 min denaturation, a cycle of denaturation at 94°C for 1 min, annealing at 63°C for 1 min and extension at 68°C for 10 min was repeated 30 times; PCR was performed using LA-PCR Kit (version 2; Takara Bio). PCR conditions for the amplification of the entire urease gene cluster using primers LA3 and LA4 have been described by Heimer et al. (2002). PCR reactions were performed in a total reaction volume of 50 μl.

Southern hybridization. To subclone DNA fragments containing a target gene, digested DNA fragments were analysed by Southern hybridization. In brief, digested DNA fragments were separated on 0.8% agarose gels and transferred onto a nylon membrane (GeneScreen Plus; NEN Life Science Products). DNA was fixed to the membrane by UV cross-linking using a GS Gene Linker UV Chamber (Bio-Rad). All DNA probes used in this study were prepared by PCR. The PCR products were separated on 2% agarose gel and extracted from the agarose gel with a QIAEXII gel extraction kit (Qiagen). DNA probes were labelled by using the PCR DIG Probe Synthesis Kit (Roche). Hybridization was carried out at 42°C with 50% formamide, and hybridized DNA was detected by alkaline-phosphatase-labeled anti-digoxigenin monoclonal antibody (Roche).

Nucleotide sequence analysis. Nucleotide sequences were determined by using the BigDye Terminator Cycle Sequencing Kit version 3.1 and an Avant-3100 automatic DNA sequencer (Applied

Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genotype</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
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<tr>
<td>RIMD0509952 (O157 Sakai)</td>
<td>stx1+ stx2+ ureD+ (inactive form) (serotype O157:H7)</td>
<td>Watanabe et al. (1996)</td>
</tr>
<tr>
<td>RIMD05091612 (Ure+/−-1)</td>
<td>stx2− ureD− (active form) (serotype Out:H+)*</td>
<td>This study</td>
</tr>
<tr>
<td>RIMD05091546</td>
<td>stx2− ureD− (active form) (serotype O157:H7)</td>
<td>This study</td>
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<td>E. coli DH5α</td>
<td>supE44 recA1 lacZU169 (delta (860 lacZD15))</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
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<td>E. coli CSH4</td>
<td>thi Δ(lac–pro) F[proAB+ lacZD15]</td>
<td>Miller (1972)</td>
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<tr>
<td>MS-1</td>
<td>E. coli DH5α with pNU3</td>
<td>This study</td>
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<td>MS-2</td>
<td>E. coli CSH4 with pNU3</td>
<td>This study</td>
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<td>O157 Sakai with pNU1</td>
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<td>MS-4</td>
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<td>O157 Sakai with pACYC184</td>
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<td>MS-6</td>
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<td><strong>Plasmids</strong></td>
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<td>Cloning vector, Amp’</td>
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</tr>
<tr>
<td>pT7-Blue T vector</td>
<td>TA cloning vector, Amp’</td>
<td>Merck</td>
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<tr>
<td>pACYC184</td>
<td>p15Aori Tet’, Cm’</td>
<td>Chang &amp; Cohen (1978)</td>
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<td>pUreAG</td>
<td>~7 kb KpnI–EcoRI fragment from Ure+/-1 in pUC19 (containing entire ure operon)</td>
<td>This study</td>
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<tr>
<td>pNU1</td>
<td>Ccl–DraI fragment from Ure+/-1 in pACYC184 (containing active ureD and Fur boxes)</td>
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<tr>
<td>pNU2</td>
<td>Ccl–DraI fragment from O157 Sakai in pACYC184 (containing inactive ureD and Fur boxes)</td>
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<tr>
<td>pNU3</td>
<td>Entire ure operon from O157 Sakai in pT7-Blue (PCR-generated, containing ureD to ureG)</td>
<td>This study</td>
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*Out, untypable with respect to O-serogroup.
Table 2. Primers

<table>
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<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>Annealing temp. (°C)</th>
<th>Product size (bp)</th>
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<td>D1</td>
<td>ATGAAACGGCAACTGGAAGAC</td>
<td>60</td>
<td>322</td>
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<tr>
<td>D2</td>
<td>ACGGATTGGGATATGGTGAG</td>
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<td>4940</td>
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<tr>
<td>LA1</td>
<td>TTGCCTAGTACCGGTTACC</td>
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<td>5000</td>
</tr>
<tr>
<td>LA2</td>
<td>TTACITACACAGCCGTGCTC</td>
<td>63</td>
<td>406</td>
</tr>
<tr>
<td>LA3*</td>
<td>TCGAGACTCTCGCCGTGATC</td>
<td>60</td>
<td>5000</td>
</tr>
<tr>
<td>LA4*</td>
<td>ACGCCAACCTGGATCTTCCCTTCTGATAA</td>
<td>63</td>
<td>406</td>
</tr>
<tr>
<td>RT1</td>
<td>ATGTCGCTGCTCAGCTTTGTT</td>
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<td>406</td>
</tr>
<tr>
<td>RT2</td>
<td>ATCTCCAGCGGTAGCCGAG</td>
<td>63</td>
<td>406</td>
</tr>
</tbody>
</table>

*Sequences described by Heimer et al. (2002).

BioSystems). DNASIS Software (version 3.7, Hitachi Software Engineering) was used to analyse the DNA sequence data. The sequences were analysed using the BLAST program available on the National Center for Biotechnology Information website.

**RT-PCR.** Bacterial strains were grown in M9 medium with 1 μM NiCl₂ until mid-exponential phase (OD₆₀₀ 0–6) and total RNA was extracted using a RNeasy Mini Kit (Qiagen). To remove the genomic DNA in extracted RNA, total RNA was treated with DNase I according to the manufacturer’s recommendations (Takara Bio). The RNA LA-PCR kit (AMV) version 1.1 (Takara Bio) was used for RT-PCR detection of ureD mRNA, according to the manufacturer’s recommendations. The oligonucleotide primers RT1 and RT2, used to amplify ureD, are described in Table 2.

**Test for urease activity.** To test for the expression of urease activity, we used urease agar base (Merck). In brief, EHEC strains were cultured in 2 ml LB broth at 37 °C with shaking for 12–16 h. Cells were inoculated onto urease agar and incubated overnight at 37 °C. EHEC strains with urease activity cause the medium to turn red. To calculate the urease activity, we used an Ammonia Test Kit (Wako Pure Chemical Industries) as described by Park et al. (2000). In brief, bacterial strains were cultured in 5 ml M9 medium with 1 μM NiCl₂ at 37 °C for 18 h, and cells were then disrupted by sonication. The supernatant was used in the urease assay, and also to determine the total protein content using the Coomassie Plus Protein Assay Reagent kit (Pierce). Urease activity was expressed as nmol urea hydrolysed min⁻¹ (mg protein)⁻¹.

**RESULTS**

**O157 Sakai expresses no urease activity in vitro**

In a previous study, we reported that most EHEC strains (21 of 22 strains tested) isolated from patients do not express urease activity, despite having the urease gene (Nakano et al., 2001). In the present study, we investigated why most EHEC strains do not express this activity. We examined the expression of urease activity by O157 Sakai under various in vitro culture conditions by changing the culture medium (LB medium, M9 medium with 1 μM NiCl₂, brain heart infusion medium or Dulbecco’s modified Eagle’s medium), the pH (pH 5, 7 or 9), the temperature (25, 30, 37 or 42 °C) and the nutrients (supplemented with Fe²⁺, Mg²⁺, Ni²⁺ or urea). In all the conditions tested, we detected no urease activity (data not shown) using the method of Park et al. (2000).

**Cloning of the entire urease gene cluster from the Ure⁺-1 strain**

To investigate why O157 Sakai does not express urease activity despite the possession of the urease genes, we first examined the size of the entire urease gene cluster (from ureD to ureG) of 120 EHEC strains out of 158 isolates from various sources. Among these strains, we found two urease-activity-positive EHEC strains. We used PCR with primers LA1 and LA2, which hybridize with the ureD and ureG genes, respectively, to estimate the length of the entire urease gene cluster. The PCR products of all strains, including the two urease-activity-positive EHEC strains, were identical in size (4940 bp) to that of O157 Sakai (data not shown). These results indicate that the 120 EHEC strains have urease gene clusters of similar size, irrespective of their urease activity.

To compare the nucleotide sequence of the urease gene cluster derived from a urease-activity-positive EHEC strain, Ure⁺-1, with that of O157 Sakai (GenBank accession no. BA000007; Hayashi et al., 2001), we cloned the ~7 kb KpnI–EcoRI fragment containing the entire urease gene cluster from the Ure⁺-1 strain (pUreAG, Table 1). The nucleotide sequence of the entire urease gene cluster revealed that its genetic organization was the same as that of O157 Sakai, containing seven genes in the order ureD, ureA, ureB, ureC, ureE, ureF and ureG, transcribed in the same direction. Except for ureD, the length of each gene from ureA to ureG in the Ure⁺-1 strain was identical to that of the corresponding gene of O157 Sakai, and the nucleotide and amino acid sequences were highly conserved between the two strains (over 99% identity). However, it was revealed that the ureD gene of the Ure⁺-1 strain had a longer ORF compared with that of O157 Sakai (Fig. 1a).

**The ureD gene of O157 Sakai has a premature stop codon**

When we compared the nucleotide sequences of ureD derived from the Ure⁺-1 strain and O157 Sakai, it was revealed that the nucleotide sequence of the intergenic region between ureD and ureA of O157 Sakai was identical.
to that of the 3’ end of ureD derived from the Ure+/-1 strain (Fig. 1a). Although the length of UreD from the Ure+/-1 strain (274 amino acid residues) was similar in size to those of other bacteria, the size of UreD from O157 Sakai (247 amino acid residues) was unique relative to those of other bacteria. The length of UreD in other bacteria described so far can be categorized into two groups: less than 220 amino acid (aa) residues and more than 260 aa residues (Fig. 1b). We compared the nucleotide sequences around the ureD genes from O157 Sakai and the Ure+/-1 strain in detail, and we found a premature stop codon in the ureD coding region of O157 Sakai (Fig. 1c). This stop codon was generated by a one-nucleotide substitution (cytosine to thymine) at the site of the 742nd nucleotide from the 5′ end of ureD (referred to as nt742 in this study) in O157 Sakai, relative to that of the Ure+/-1 strain.

We investigated the nucleotide sequences of ureD in 158 EHEC strains (see Methods). We used PCR with primers D1 and D2 (Table 2, Fig. 1a), which hybridized on either side of the premature stop codon in ureD of O157 Sakai, and analysed the nucleotide sequences of the amplified products. All the tested urease-activity-negative strains isolated from different sources (human, bovine, deer, sheep and seagull), had sequences identical to that of O157 Sakai (data not shown). We also analysed the nucleotide sequence of ureD in EHEC strain EDL933 (GenBank accession no. AE00517H; Perna et al., 2001) was identical to that of O157 Sakai. These results indicate that ureD containing a premature stop codon, as found in O157 Sakai, is common among urease-activity-negative EHEC strains. Therefore, we speculated that the presence or absence of the premature stop codon could be relevant to the expression of urease activity in EHEC strains.
**ureD is transcribed in O157 Sakai in vitro**

To investigate whether **ureD** of O157 Sakai was transcribed in vitro, we carried out RT-PCR specific for **ureD**, using primers RT1 and RT2 (Table 2) to detect **ureD** mRNA. The mRNA was detected in O157 Sakai, as it was in the Ure⁻¹ strain (Fig. 2). This result indicates that the **ureD** gene is transcribed in O157 Sakai irrespective of the lack of urease activity.

**O157 Sakai complemented with **ureD** from the Ure⁺⁻¹ strain expresses urease activity**

To examine whether the premature stop codon of **ureD** is responsible for the lack of urease activity in O157 Sakai, we introduced **ureD** from the Ure⁺⁻¹ strain into O157 Sakai (MS-3) by transformation with a **Cla**–**Dra** fragment containing **ureD** and its upstream region from the **Kpn**–**Eco** fragment of **pUreAG** (pNU1) (Table 1). The complementation elevated the urease activity of O157 Sakai (Fig. 3). Complementation with the **Cla**–**Dra** fragment containing **ureD** from O157 Sakai (pNU2) (MS-4) or pACYC184 (MS-5) (Table 1) did not affect the urease activity (Fig. 3). The results suggest that the lack of expression of urease activity by O157 Sakai is due to its **ureD**.

Recently, Heimer et al. (2002) reported that recombinant **E. coli** DH5α, carrying the entire PCR-generated urease gene cluster of strain EDL933 (pEDL933), showed urease activity. Because the **ureD** gene of strain EDL933 was identical in nucleotide sequence to that of O157 Sakai, we prepared recombinant **E. coli** DH5α (MS-1) carrying the entire PCR-generated urease gene cluster of O157 Sakai (~5 kb), amplified with primers LA3 and LA4 (pNU3) (Tables 1 and 2). The urease activity of MS-1 was 700 ± 90 nmol NH₃ min⁻¹ (mg protein)⁻¹ (mean ± SD, n = 3), which is similar to that of recombinant **E. coli** DH5α carrying pEDL933, as described by Heimer et al. (2002). To explain why the urease gene cluster of O157 Sakai expresses urease activity in **E. coli** DH5α despite the presence of the premature stop codon in the **ureD** gene, we speculated that this phenomenon could be due to the amber suppressor phenotype of **E. coli** DH5α. Because the premature stop codon of **ureD** from urease-activity-negative EHEC strains, including O157 Sakai, was the amber stop codon TAG (Fig. 1c), it is possible that the amber suppressor phenotype of **E. coli** DH5α, caused by a **supE44** mutation (Miller, 1972), can read through the amber stop codon in **ureD**. To confirm this hypothesis, the recombinant plasmid pNU3 was introduced into **E. coli** CSH4 (MS-2), which does not have the amber suppressor phenotype (Miller, 1972), and the urease activity of MS-2 was analysed. As expected, the urease activity of MS-2 [65 ± 20 nmol NH₃ min⁻¹ (mg protein)⁻¹] was much lower than that of MS-1. We detected **ureD** mRNA in MS-2, as well as in MS-1 (Fig. 2). These results suggest that the urease activity of MS-1 or pEDL933 in **E. coli** DH5α is due to the amber suppressor phenotype of the **E. coli** host strain.

**DISCUSSION**

In a previous study, we reported that the urease gene was specifically associated with EHEC strains isolated from patients irrespective of their serogroups; however, most of the EHEC strains showed no urease activity (Nakano et al., 2001). The present study demonstrated a correlation between the ureolytic phenotype of EHEC and the nucleotide sequence of **ureD**, which serves as a chaperone protein in maintaining protein conformation or assists in binding of nickel ions (Park et al., 1994; Park & Hausinger, 1995). When a thymine residue was present at nt742 of **ureD**, no urease activity was detected in EHEC strains. In contrast, when a cytosine residue was present at nt742 of
ureD, EHEC was capable of hydrolysing urea. Our current study demonstrated that the presence or absence of the premature stop codon at nt742 of ureD is critical to the production of active urease. Thus, the urease activity of EHEC strains depends on a one-base difference in the nucleotide sequence of ureD.

Although we detected substantial urease activity in MS-1 [700 ± 90 nmol NH₃ min⁻¹ (mg protein)⁻¹], we detected only low activity in MS-2 [65 ± 20 nmol NH₃ min⁻¹ (mg protein)⁻¹]. These results indicate that the urease activity observed in MS-1 was due to the amber suppressor phenotype of the host strain. In this strain, the amber stop codon at nt742 of ureD could be read through and translation could proceed to next ochre or opal stop codon (Garen, 1968). This resulted in the production of a full-length form of UreD, leading to the formation of active urease.

Regulation of the urease genes in EHEC is not well understood. Recently, Heimer et al. (2002) reported that the putative Fur boxes are present upstream of the urease operon, and that Fur is involved in the regulation of expression of the urease activity. Except for the Fur boxes, no other regulatory elements are known in the upstream region of the EHEC urease operon. From the results showing that the EHEC ure operon introduced in E. coli DH5α produced active urease, Heimer et al. (2002) postulated that an unidentified trans-acting factor also regulates ure expression. The phenomenon observed by those authors can be explained by our finding in the present study, i.e. the production of active urease in E. coli DH5α is due to the amber suppressor phenotype of the E. coli DH5α strain.

Previous studies showed that very few urease-activity-positive EHEC strains are isolated from patients or cattle (Nakano et al., 2001; Tutenel et al., 2003). Nevertheless, the present study revealed that EHEC strains retain the complete urease gene cluster, irrespective of their urease activities. The nucleotide sequence at nt742 of ureD in O157 Sakai is well conserved in the urease-activity-negative EHEC strains tested. At present, it is unclear why most EHEC strains carry the inactive form of ureD. Synthesized bacterial urease accumulates in the cytoplasm (Mobley et al., 1995). If EHEC strains were to continuously synthesize the active form of urease, ammonia produced by hydrolysis of urea could accumulate in the cytoplasm, leading to the elevation of pH in bacterial cells. Thus, most EHEC strains might retain the short form of ureD to prevent the damage of bacterial cells by alkaline conditions caused by ammonia. Since it is known that urease is essential for some bacteria to use urea as the nitrogen source (Chen et al., 2000), producing active urease could be beneficial for EHEC strains under certain circumstances in natural environments. One possible explanation is that active urease would be produced by EHEC strains by a mutation at the nt742 site of ureD. The putative mutation at nt742 might be spontaneous or caused by a so far unknown genetic mechanism in the organism. Alternatively, under certain circumstances, the amber stop codon at nt742 could be somehow read through in EHEC strains. These possibilities are to be explored in the future.

Phenomena which turn on/off the ureolytic activity of bacteria by a one-base alteration have been reported in Yersinia pestis and Helicobacter pylori (Hansen & Solnick, 2001; Sebbane et al., 2001). In Y. pestis, ureolytic activity depends on the number of guanine (G) residues (poly G tract) in ureD, and in H. pylori it depends on a one-base insertion in ureA. It is not known whether a specific nucleotide substitution at nt742 of ureD occurs in EHEC strains. If it does occur, the mechanism should differ from those of Y. pestis and H. pylori because the type of sequence alteration is obviously different.

**ACKNOWLEDGEMENTS**

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