Urease activity of enterohaemorrhagic *Escherichia coli* depends on a specific one-base substitution in *ureD*

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The authors previously reported that most enterohaemorrhagic *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, *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 *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 *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 *Escherichia coli* strain, DH5 α , 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 *ureD*.

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INTRODUCTION

Enterohaemorrhagic *Escherichia coli* (EHEC) is a food-borne pathogen that can cause severe clinical manifestations such as haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) (Verwey *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, 1991). EHEC strains produce a potent Shiga toxin (Stx) responsible for the major symptoms of HC and HUS (Verwey *et al.*, 2000). Other possible virulence determinant genes 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, *ureA*, *ureB* and *ureC*, and four accessory genes, *ureD*, *ureE*, *ureF* and *ureG* (Hayashi *et al.*,

2001; Perna *et al.*, 2001). Based on the genome sequence, the urease genes are most similar to those of *Klebsiella aerogenes* (Mulrooney & Hausinger, 1990; Lee *et al.*, 1992).

Recently, we reported that, among pathogenic *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 *ureD* gene.

Abbreviations: EHEC, enterohaemorrhagic *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 faeces, 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-labelled 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

Strain/plasmid	Genotype	Reference
<i>E. coli</i> strains		
RIMD0509952 (O157 Sakai)	<i>stx1</i> ⁺ <i>stx2</i> ⁺ <i>ureD</i> ⁺ (inactive form) (serotype O157:H7)	Watanabe <i>et al.</i> (1996)
RIMD05091612 (Ure ⁺ -1)	<i>stx2</i> ⁺ <i>ureD</i> ⁺ (active form) (serotype Out:H ⁻)*	This study
RIMD05091546	<i>stx2</i> ⁺ <i>ureD</i> ⁺ (active form) (serotype O157:H7)	This study
<i>E. coli</i> DH5α	<i>supE44</i> <i>recA1</i> <i>lacZU169</i> (φ80 <i>lacZAM15</i>)	Yanisch-Perron <i>et al.</i> (1985)
<i>E. coli</i> CSH4	<i>thi</i> Δ(<i>lac-pro</i>) F'[<i>proAB</i> ⁺ <i>lacZ</i>]	Miller (1972)
MS-1	<i>E. coli</i> DH5α with pNU3	This study
MS-2	<i>E. coli</i> CSH4 with pNU3	This study
MS-3	O157 Sakai with pNU1	This study
MS-4	O157 Sakai with pNU2	This study
MS-5	O157 Sakai with pACYC184	This study
MS-6	O157 Sakai with pNU3	This study
Plasmids		
pUC119	Cloning vector, Amp ^r	Yanisch-Perron <i>et al.</i> (1985)
pT7-Blue T vector	TA cloning vector, Amp ^r	Merck
pACYC184	p15Aori Tet ^r , Cm ^r	Chang & Cohen (1978)
pUreAG	~7 kb <i>KpnI</i> – <i>EcoRI</i> fragment from Ure ⁺ -1 in pUC119 (containing entire <i>ure</i> operon)	This study
pNU1	<i>Clal</i> – <i>DraI</i> fragment from Ure ⁺ -1 in pACYC184 (containing active <i>ureD</i> and <i>Fur</i> boxes)	This study
pNU2	<i>Clal</i> – <i>DraI</i> fragment from O157 Sakai in pACYC184 (containing inactive <i>ureD</i> and <i>Fur</i> boxes)	This study
pNU3	Entire <i>ure</i> operon from O157 Sakai in pT7-Blue (PCR-generated, containing <i>ureD</i> to <i>ureG</i>)	This study

*Out, untypable with respect to O-serogroup.

Table 2. Primers

Primer	Sequence (5'–3')	Annealing temp. (°C)	Product size (bp)
D1	ATGAAACGCAACTGGAAGAC	60	322
D2	ACGGATTCGGGATAGTTAAG		
LA1	TTGCTCAGTACCGCGTTACC	63	4940
LA2	TTACTTACCCAGCATGCCCT		
LA3*	TCGGAGCTCTCTGCCTGATTCAGTGGATAA	60	5000
LA4*	AACGCCAACTTGGATCCTTCCTTCTGATAA		
RT1	ATGTCGGTCTGCTCACTGTT	63	406
RT2	ATCTCCAGACGGTTAGCGAG		

*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 μ M NiCl_2 until mid-exponential phase (OD_{600} 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 μ M NiCl_2 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 $\text{nmol urea hydrolysed min}^{-1} (\text{mg protein})^{-1}$.

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 μ M NiCl_2 , 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 Fe^{2+} , Mg^{2+} , Ni^{2+} 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 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 *Kpn*I–*Eco*RI 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

Fig. 1. Characterization of *ureD* from O157 Sakai and the Ure⁺-1 strain. (a) Homology of nucleotide and amino acid sequences of *ureD* of O157 Sakai (Sakai) and the Ure⁺-1 strain (Ure⁺). Thick and thin arrows indicate the direction of transcription and locations of primers, respectively. (b) Size variation of UreD from various bacterial species. GenBank accession numbers: CAC85465 (*L. esculentum*), BAB43386 (*S. aureus*), AAL52836 (*B. melitensis*), Q09063 (*K. aerogenes*), BAB21064 (*R. capsulatus*), AAD13726 (*A. naeslundii*), A43719 (*Prot. mirabilis*), AAC78638 (*Y. pestis*), AAO05508 (*S. epidermidis*), AAG08249 (*Ps. aeruginosa*), BAB13785 (*V. parahaemolyticus*), CAB81941 (*C. glutamicum*), Q56563 (*U. urealyticum*), CAE19425 (*Proch. marinus*) and P52316 (*Y. pseudotuberculosis*). (c) Nucleotide and deduced amino acid sequences of *ureD*. Sakai, O157 Sakai; Ure⁺, Ure⁺-1 strain. Thick arrows indicate the direction of transcription. The arrowhead and bold letters indicate the difference in the nucleotide sequences derived from O157 Sakai and the Ure⁺-1 strain.

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 sequences of *ureD* in another urease-activity-positive EHEC strain (RIMD05091546) isolated from a human patient. This strain had an identical sequence to that of the Ure⁺-1 strain (data not shown). Furthermore, 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.

Fig. 2. RT-PCR specific for *ureD*. M, DNA size marker; Sakai, O157 Sakai; Ure⁺-1, a urease-positive strain; MS-1, *E. coli* DH5 α (pNU3); MS-2, *E. coli* CSH4(pNU3); DH5 α , *E. coli* DH5 α , used as the negative control. Strains were cultured in M9 medium with 1 μ M NiCl₂.

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 *Clal*-*DraI* fragment containing *ureD* and its upstream region from the *KpnI*-*EcoRI* fragment of pUreAG (pNU1) (Table 1). The complementation elevated the urease activity of O157 Sakai (Fig. 3). Complementation with the *Clal*-*DraI* 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* DH5 α , 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* DH5 α (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 $\pm$ SD, $n=3$), which is similar to that of recombinant *E. coli* DH5 α carrying pEDL933, as described by Heimer *et al.* (2002). To

Fig. 3. Urease activity of recombinant O157 Sakai after complementing with *ureD* from O157 Sakai or the Ure⁺-1 strain. Sakai, O157 Sakai; Ure⁺-1, this strain used as a positive control; MS-3, O157 Sakai with pNU1; MS-4, O157 Sakai with pNU2; MS-5, O157 Sakai with pACYC184 used as the negative control. Bacterial strains were cultured in M9 medium with 1 μ M NiCl₂. Results are the means of three independent experiments $\pm$ SD.

explain why the urease gene cluster of O157 Sakai expresses urease activity in *E. coli* DH5 α 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* DH5 α . 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* DH5 α , 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* DH5 α 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 NH_3 min^{-1} (mg protein) $^{-1}$], we detected only low activity in MS-2 [65 ± 20 nmol NH_3 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* DH5 α 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* DH5 α is due to the amber suppressor phenotype of the *E. coli* DH5 α 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.

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