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A homolog of the O157 urease-encoding O island 48 is present in porcine O149:H10 enterotoxigenic *Escherichia coli*

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Abstract – The relationship of the urease operon in the highly virulent O149 porcine enterotoxigenic *Escherichia coli* (ETEC) strain Ro8 to a genomic island (GI) homologous to O island (OI) 48 of O157 enterohemorrhagic *E. coli* (EHEC) strain EDL933 was investigated. Eighty-four of 84 O149:H10 strains were urease positive whereas 44 of 44 O149:H43 porcine ETEC strains were urease-negative. Seventeen of 17 O149:H10 strains that were tested possessed the OI-48 homolog whereas 24 of 24 O149:H43 strains lacked this OI. Transposon insertions in *lipB* or *guaA* genes in strain Ro8 eliminated urease activity while insertions in the *caiF* gene increased urease activity. When the O149 *ure* operon was cloned on a high copy number plasmid, urease expression was increased approximately 11-fold in Ro8 and 83-fold in O157 strain EDL933 compared with that in the wild type Ro8. The O149 urease activity was expressed despite the presence of the same premature stop codon in *ureD* that is present in *ure*+ O157:H7 strains that are urease-negative. The *ure* operon in Ro8 consists of 4 893 nucleotides with 99% identity with the *ure* operons in EHEC O157:H7 strains EDL933 and Sakai, and is part of a GI similar to GI-48 of strain EDL933. This OI, designated OI-48_{149}, is inserted in the *serX* tRNA gene in strain Ro8 and contains genes for urease, tellurite resistance, *iha* and an AIDA-I-like adhesin. The presence of a homolog of the O157:H7 OI-48 in highly virulent O149 porcine ETEC suggests that this OI may contribute to establishment of the bacteria in the intestine.

urease / O149 / porcine enterotoxigenic *E. coli* / OI-48 / genomic island

1. INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) are a major cause of diarrhea in both neonatal and recently-weaned pigs [26]. Two major virulence factors of ETEC are fimbriae that mediate adherence to enterocytes, and enterotoxins that induce hypersecretion of electrolytes and fluid into the lumen of the intestine. Little is known of other contributors to virulence. Porcine ETEC belong to a limited number of O serogroups, of which O149 has been dominant worldwide for decades [6, 12, 14, 18, 45, 46]. In 1997, there was a dramatic increase in frequency and severity of post-weaning diarrhea (PWD) in pigs in Ontario, Canada, that was associated with O149 ETEC [21]. The isolates associated with severe disease were mostly frequently O149:H10:F4 organisms that had the genes for enterotoxins LT, STa, STb, and EAST-1 [31]. The isolates from earlier years and 9.6% of the recent isolates were O149:H43:F4 that lacked the gene for STa. In addition, all but one of the recent O149 ETEC isolates, but none of the less recent isolates, had a delayed urease-positive phenotype that was evident after incubation for 2–3 days on Christensen's urea agar.

This delayed urease-positive phenotype was reported previously in the original description of F4-positive O149 isolates [34] and in

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association with F4-negative O149 ETEC that caused outbreaks of PWD in pigs in Denmark [22], but the genotype has never been investigated. As well, urease production was noted in ETEC recovered from PWD that were O141:K85 (56%), O147:K89 (50%), O8:K87 (14%), O138:K81 (14%) or nontypeable strains (14%) [22].

There are two distinct types of the urease operon in *E. coli* [4], a plasmid-encoded urease operon that is highly homologous to urease operon in plasmids in *Proteus mirabilis*, *Providencia stuartii,* and certain *Salmonella* serovars, and a chromosomal cluster which is homologous to clusters in *Klebsiella, Enterobacter, Citrobacter,* and *Serratia*. A chromosomal urease operon is present in enterohemorrhagic *E. coli* (EHEC) including strains of O157:H7, O5, O26, O111 and O145 [10, 17, 27], but, except for the O5 strains, urease activity is not usually expressed in vitro by EHEC strains. Friedrich et al. [10] reported that only one of 58 O157:H7 strains had a urease-positive phenotype although all 58 strains had the seven genes of the *ure* operon. Interestingly, sorbitol-positive EHEC O157:NM lacked the *ure* genes.

In most organisms the urease operon consists of *ureA, ureB, ureC,* which encode the structural subunits of the enzyme, and *ureD, ureE, ureF* and *ureG,* whose products are thought to be involved in activation and incorporation of nickel in the apoenzyme [2]. In *P. mirabilis* expression of the urease genes is regulated by the AraC-like transcriptional activator, UreR, whose gene is 414 bp upstream of *ureD*, the first gene in the cluster [13, 42]. In *K. pneumoniae*, the *ure* genes are regulated by the nitrogen regulatory cascade and NAC, a Lys R-like transcriptional regulator that is a member of this cascade, activates transcription of *ureD* by binding to its promoter [5].

Urease is a well characterized virulence factor of many uropathogens, a number of respiratory pathogens, and one gastric pathogen, *Helicobacter pylori* [2]. There is little information on urease in ETEC. The goals of this research were to characterize the urease operon in an O149 porcine ETEC and determine whether these genes were part of a genomic island (GI) similar to OI-48 in O157 EHEC EDL933.

2. MATERIALS AND METHODS

2.1. Bacterial strains, plasmids, and growth conditions

E. coli strains examined in this study consisted of 94 O149:F4 porcine ETEC strains isolated from outbreaks of severe diarrhea in Ontario pigs between 1998 and 2001 (R strains), 36 O149:F4 porcine ETEC isolated in earlier years (1977–1985) (O strains) [31], 200 non-O149 ETEC strains, and 100 non-ETEC strains isolated from the feces of healthy pigs during 2000–2003. The O149 strains were identified by agglutination in anti-O149 serum prepared in rabbits and by PCR amplification of the *fliC* genes that identified the H10 and H43 antigens [31]. Where there was uncertainty about the serotype, the strain was also serotyped by the *E. coli* reference serotyping laboratory at the Laboratory for Foodborne Zoonoses (Public Health Agency of Canada, Guelph, Ontario). The O149 ETEC strains were all hemolytic. Pulsed-field gel electrophoresis (PFGE) of genomic DNA showed that the O149:H10 ETEC and the O149:H43 ETEC were distinctly different and that there was marked heterogeneity in PFGE patterns within each of these two groups (Noamani B. and Gyles C., unpublished results). The high virulence of the O149:H10 ETEC strain R08 was shown in experimental infections of 36 pigs (Boerlin P. and Gyles C., unpublished results).

The *E. coli* strains (Tab. I) were grown in Luria-Bertani (LB) broth or on LB plates. When antibiotic selection was necessary, the growth medium was supplemented with ampicillin (100 μg/mL), kanamycin (50 μg/mL), gentamicin (25 μg/mL) or nalidixic acid (50 μg/mL). Reference *E. coli* strain C600 (K12) was used as a negative control and O157:H7 strains EDL933 and Sakai (Tab. I) were used as positive controls. All strains were stored at –70 °C in LB broth containing 15% glycerol. *E. coli* strain DH5α was used as a host for cloning.

2.2. Genetic manipulations

All genetic manipulations were performed by standard methods as described by Sambrook et al. [39]. Plasmid DNA was purified with a Plasmid Mini kit (Qiagen, Mississauga, ON, Canada).

Table I. *E. coli* strains and plasmids.

Strain/Plasmid	Genotype	Source/Reference
E. coli strains		
Ro8	O149:H10 wild-type ETEC strain	[31]
Ro8N	Spontaneous nalidixic acid resistant mutant of Ro8	This study
DH5α-pVPL	DH5α with plasmid pVPL	This study
DH5α-pVPH	DH5α with plasmid pVPH	This study
DH5α-pVPHZ	DH5α with plasmid pVPHZ	This study
EDL933-pVPH	O157:H7 strain EDL933 with plasmid pVPH	This study
Sakai-pVPH	O157:H7 strain Sakai with plasmid pVPH	This study
Ro8M	Ro8 *ureC* mutant (Km^r)	This study
Ro8M-pVPH	Ro8 *ureC* mutant complementes with plasmid pVPH (Km^r, Ap^r)	This study
DH5αλpir	Φ80dlacZΔM15 Δ(lacZYAargF) U169 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1/λpir	[9]
SM10λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu (Km^R) [λpir]	[24]
EDL933	$stx1^+$ $stx2^+$ *ure* cluster+ (2 copies, inactive) (serotype O157:H7)	[32]
Sakai	$stx1^+$ $stx2^+$ *ure* operon+ (1 copy, inactive) (serotype O157:H7)	[44]
DH5α	*supE44 lacU169(Φ80 lacZΔM15) hsdR17recA1endA1*	Invitrogen
C600 (K12)	F- *thr leu thi lac tonA*	[33]
Plasmids		
pUC18/pUC18K	Cloning vector, high-copy (Ap^r)	Fermentas/ [23]
pBluescript II SK	Cloning vector, high-copy (Ap^r)	Fermentas
pGEM-T	TA cloning vector, high copy (Ap^r)	Promega
pEXT-22	Cloning vector, low copy (Km^r)	[7]
pRE107	Suicide vector, *oriT oriV sacB* Ap^r	[8]
pVPureC	Suicide vector pRE107 (Ap^r, Km^r) with the *ureC* gene	This study
pLOF-Km	Tn*10*-based delivery plasmid with Km^r (Ap^r)	[19]
pVPL	Low copy plasmid pEXT22 with the entire *ure* operon from Ro8	This study
pVPH	High copy plasmid pGEM-T with the entire *ure* operon from Ro8 (PCR-generated, with upstream and downstream primers, Heimer et al. [17])	This study
pVPHZ	High copy plasmid pGEM-T with the *Z1580* gene + the entire *ure* operon from Ro8 (PCR-generated, with primer Zf and downstream primer from Heimer et al. [17])	This study

DNA fragments were extracted from agarose gel slices and purified with the Geneclean III Kit (BIO101 Inc., La Jolla, CA, USA). DNA ligations and restriction endonuclease digestions were performed with enzymes supplied by New England Biolabs (Mississauga, ON, Canada) or Amersham Pharmacia Biotech (Piscataway, NJ, USA). DNA transformations were performed by the heat shock method described by Nishimura et al. [30].

2.3. Detection of urease activity on agar media

E. coli strains were examined for urease activity on urea agar, which contains the pH-sensitive, colorimetric indicator phenol red [3]. A modified MacConkey-urea medium (MUM) was developed to facilitate detection of urease-negative mutants as these mutants were not detectable in the presence of large numbers of urease-positive colonies that changed the pH of the entire Christensen's urea agar on a plate. Each litre of MUM contained urea (20 g), NaCl (5 g), KH_2PO_4 (2 g), Bacto Peptone (1 g), glucose (1 g), neutral red (0.03 g), and bile salts (1.5 g).

2.4. Measurement of urease activity

Urease enzymatic activity was determined essentially as described by Heimer et al. [17]. The strains were each grown from a single colony in 5 mL of M9 minimal medium with casamino acids overnight at 37 °C. Bacteria were harvested by centrifugation (12 000 × *g*, 2 min, 4 °C) and

washed twice with 50 mM HEPES buffer (pH 7.5). Bacteria were kept on ice and lysed by sonication to release cytosolic proteins, including urease. The lysates were centrifuged (12 000 × *g*, 2 min, 4 °C) and the supernatants were placed on ice. Protein concentrations of the extracts were determined using a 30 min bicinchoninic acid assay method (Pierce Chemical Company, Rockford, IL, USA), according to the manufacturer's instructions with bovine serum albumin as the standard.

2.5. Distribution of the *ureC* gene in O149 ETEC

Colony hybridizations were performed according to the procedure detailed previously [36]. A PCR-digoxigenin-11-dUTP (DIG) probe was prepared and detected following the manufacturer's recommendation (The DIG system user's guide for filter hybridization, Boehringer-Mannheim, Gmbh, Germany). A DIG-labeled PCR probe was made using ETEC O149 strain Ro8 chromosomal DNA as template and specific primers for the *ureC* gene (Tab. II). The blots were hybridized with the DNA probe solution containing 50% formamide at high stringency and washed at 65 °C.

2.6. Cloning and sequencing the ETEC O149 urease operon

The *ure* operon from ETEC O149 strain Ro8 was amplified using Ro8 chromosomal DNA and primers (Tab. II) to sequences 44 bp upstream of the first potential *ureD* start site to 53 bp downstream of the stop codon for *ureG* in strain EDL933 [17]. PCR amplifications were carried out in a 50-µL total volume with 5 µL of genomic DNA (purified by Qiagen Genomic-Tip) from strain Ro8, 0.5 µM of each primer, and Platinum PCR SuperMix High Fidelity (Invitrogen Life Technologies, Carlsbad, CA, USA). Amplified fragments of approximately 5 kb were cloned into pGEM-T Easy and pEXT-22, producing pVPH and pVPL, respectively, and transformed into *E. coli* DH5α (Stratagene, La Jolla, CA, USA). Recombinant strains, named DH5α-pVPH and DH5α-pVPL, respectively, were examined for urease expression on urea agar and the insert DNA was sequenced.

2.7. Construction of a urease deletion mutant

A urease deletion mutant of ETEC O149 strain Ro8N (Nal^R) was constructed by allelic exchange using the suicide vector pRE107 [15]. Plasmid pVPureC (Ap^r), was constructed by amplifying fragments of the urease operon from ETEC O149 strain Ro8N using primer pair UreUP-F (*Xba*I) and UreUP-R (*Kpn*I) and primer pair UreDW-F (*Kpn*I) and UreDW-R (*Sac*I) (Tab. II), generating PCR fragments of 951 bp and 912 bp, respectively. The two amplified DNA fragments were cloned, consecutively, into pRE107. A kanamycin resistance cassette derived from pUC-KM was then cloned into the *Kpn*I site near the middle of the amplified fragments giving rise to pVPureC (Ap^rKm^r). This plasmid was sequenced to determine the orientation of the kanamycin resistance cassette and to confirm its in-frame insertion. The pVPureC plasmid was electroporated into *E. coli* DH5αλpir, then transferred to strain Ro8N by conjugal mating. A urease-negative mutant strain named Ro8M (Nal^r;Δ*ure*- Kan^r) with 746 bp deleted (from position 2722 to 3468, GenBank AE005308) was generated by allelic exchange using kanamycin and sucrose selection as previously described [15]. Strain Ro8M-pVPH was created by complementing Ro8M with pVPH.

2.8. Transposon mutagenesis and colony selection

To create urease mutants, the suicide vector pLOFKm, a Tn*10*-based transposon vector with a gene for kanamycin resistance (Kan^r) was mobilized from *E. coli* SM10λ*pir* into *E. coli* Ro8N by conjugation as described by Herrero et al. [19]. Urease hyperproducers and transformants that did not express urease activity were identified on Christensen's urea agar plus kanamycin and nalidixic acid and on modified urea medium (MUM) plus kanamycin and nalidixic acid.

Transposon insertion locations of urease-negative mutants were determined by PCR, IPCR and sequencing. In order to eliminate urease negative mutants in which the Tn*10* transposon was located in the urease operon, PCR was performed using primers ureUp and ureDown (Tab. II), which amplify the entire urease operon. Mutants with an intact urease operon, but no urease production were selected for further characterization using inverse PCR amplification. Chromosomal DNA (0.5 µg) was digested with *Rsa*I and then self-ligated in a 200 µL reaction volume with 80 U of T4 DNA ligase (400 U/µL; New England Biolabs, Mississauga, ON, Canada). Ligated DNA preparations were used as templates in an inverse PCR with

Table II. Primers used in the study.

Oligonucleotides	Sequence 5' – 3'	Reference
ureUP	TCGGAGCTCTCTGCCTGATTCACTGGATAA_	[17]
ureDW	AACGCCAACTTGGATCCTTCCTTCTGATAA	[17]
Z-F (*Xba*I)	CCGC TCTAGATACAACACCACTAACATCTT	This study
ureC-F	AGGCCATGGGACGCGTCGGA	This study
ureC-R	ACTTCTCCGACGCGTCCCAT	This study
ureUP-F(*Xba*I)	CCGTCTAGAGCATGTCCTGAGTCGCGAGCA	This study
ureUP-R(*Kpn*I)	CCGTGGTACCTACAGGTTGTGGCGTTAGAA	This study
ureDW-F (*Kpn*I)	CCTGGTACCTGAAGTCGGCTCGATTGAAGT	This study
ureDW-R (*Sac*I)	CCGGAGCTCTGCTCCTGATCGTGATTATGG	This study
terA-F (O43/48F)	AACCGGCTGAAACCTGATGTC	[25]
terA-R (O43/48R)	AATTGCGCCGTTTTCGTTTAC	[25]
iha-F	GCGGAGGCTCTGAGATCAGT	This study
iha-R	ACATCCTGCTCCGACGCCTT	This study
AIDA-F	TCAGAACAGAGGTTGCCGGTATGTC	This study
AIDA-R	GAACAGCAATGAGCGTCCTT	This study
O43 left junction fwd	CGCCATACCGTCAGCCGTCTTA	[25]
O43 left junction rev	CATTTCCCCCGCCCCTTTACT	[25]
O43 right junction fwd	GGGACCGGTGGGGATTTCAT	[25]
O43 right junction rev	CCCCCTCACCGCCAGATTAT	[25]
O48 left junction fwd	GGGGGACCGCCTGAAATAAATCT	[25]
O48 left junction rev	ACTCGCCCCGGAATGTCACTG	[25]
O48 right junction fwd	CTGCGGCTGCTGGCTGATG	[25]
O48 right junction rev	GTGAGGTGTCCGAGTGGCTGAAG	[25]
LA-F	AAGACGTAATGCTGGATTGG	This study
LA-R	TTGATGACGTTCTCACTGCCGGTAG	This study
LB-F	GCGTATCCTGTGCTGCTCAGAATGT	This study
LB-R	CGGTACTGAGCAATCTTACG	This study
LC-F	AACTGACCTTGCACCATACG	This study
LC-R	GACATCCATTCACCAACTTACCGGAG	This study
LD-F	GACCAGGAATACGTTATCAGGCGTC	This study
LD-R	AATTGCGCCGTTTTCGTTTAC	This study
LE-F	AACCGGCTGAAACCTGATGTC	This study
LE-R	ACATCCTGCTCCGACGCCTT	This study
LF-F	GTAACTGGCTGGCATTCCTC	This study
LF-R	TTCCATCAGATGTCCTTCCTGCTCC	This study
LG-F	ACAACCGTCCTCCGTATCCTGTCAT	This study
LG-R	CTGCTGCGAGGCTTCATCAATA	This study
LH-F	CCATTGATGATTCCGGAGAGAAGG	This study
LH-R	TGATGTGACAGCAGCAAGAGACAGC	This study
LI-F	TCAGAACAGAGGTTGCCGGTATGTC	This study
LI-R	GTCAGGTGTCCGAGTGGCTGAAG	This study

specific transposon primers facing outwards from the kanamycin resistance gene sequence. Finally, amplified DNA fragments were directly sequenced using the same primers.

Chromosomal DNA of transconjugants was extracted using a GenomicPrep Cells and Tissue DNA Isolation kit (Amersham Biosciences Ltd, Buckinghamshire, UK), digested with restriction enzymes *Eco*RI, *Kpn*I and *Pst*I, transfered to a nylon type membrane and hybridized with a PCR-DIG probe made by using pLOF-KM as template and specific primers for the kanamycin resistance gene. Hybridizations were performed as described previously [36].

2.9. A genomic island in recent O149 porcine ETEC

To look for selected genes that might be present in a potential GI in the O149 ETEC strain Ro8, PCR amplifications were performed using specific primers for genes encoding tellurite resistance (*terA*), IrgA homologue adhesin (*iha*), and adhesin involved in diffuse adherence (AIDA). Primer pairs are described in Table II and all PCR reactions were carried out as described by Morabito et al. [25]. Location of the site of insertion of the GI in O149 ETEC strain Ro8 was determined by PCR amplification, using primer pairs described by Morabito et al. [25] that selected for the O43 and O48 left and right junctions (Tab. II). In order to determine continuity among the genes in the potential O149 GI, nine long-range PCR were performed using specific primers designed on the basis of the sequence of OI-48 of O157:H7 strain EDL933. The predicted PCR product sizes were based on sequences from EHEC O157 strain EDL933. Sequencing was carried out to identify the terminal regions of the fragments that were amplified.

2.10. Tellurite resistance test

Tellurite resistance tests were performed as previously described by Taylor et al. [41]. Briefly, cultures were grown to a concentration of 10^8 cells/mL in BHI broth. A 100 µL volume of an overnight culture was diluted in 5 mL of phosphate-buffered saline (Oxoid, Nepean, Ontario, Canada), and 10 µL of this dilution was spotted onto plates containing serial two-fold concentrations of potassium tellurite (Sigma Chemical Co., St-Louis, MO, USA) ranging from 1 µg/mL to 1 024 µg/mL. The plates were incubated at 37 °C overnight. The minimum inhibitory concentration (MIC) was the lowest concentration of potassium tellurite that prevented visible bacterial growth.

2.11. DNA sequencing and sequence analysis

Nucleotide sequencing was performed by the Guelph Molecular Supercentre with the ABI-prism fluorescent Big Dye Terminators system using universal vector primers or synthetic oligonucleotides designed on the basis of preceding sequences. Similarities and identities between the DNA in the pathogenicity island (PAI) and the translated protein sequences were made by comparisons with sequences in the GenBank genetic sequence database using the BLAST programs accessed from the National Center for Biotechnology Information (NCBI).

2.12. Nucleotide sequence accession number

The sequence data reported here have been submitted to GenBank and have been assigned accession number EU395786.

3. RESULTS

3.1. Urease activity on Christensen's urea agar plates

Urease activity of all the porcine *E. coli* strains was examined on Christensen's urea agar. Urease-positive strains all showed a delay of 48–72 h in their ability to change the color of Christensen's urea agar. Eighty-four of the 94 recent O149 ETEC strains were H10 and urease-positive, whereas the remaining ten recent isolates and 34 of the 36 less recent isolates were H43 and urease-negative. The remaining two less recent isolates consisted of an O149:H10 and an O149:NM strain that were also urease-negative. Among the other porcine strains, only two of the 200 non-O149 ETEC and none of the 100 normal flora *E. coli* were urease-positive.

3.2. Presence of *ureC* gene in O149 ETEC detected by colony blot

To investigate the presence of the *ureC* gene in porcine ETEC O149, the collection of 130 O149 strains was screened by colony blot with a *ureC*-specific probe. The results were identical to those obtained for expression of urease on Christensen's urea agar.

3.3. Sequence analysis of the urease operon

The urease operon of the ETEC O149 strain Ro8 consists of 4 893 nucleotides with a genetic organization that is identical to the urease operon in EHEC strains EDL933 and Sakai (*ureDABCEFG*). The premature stop codon in *ureD* of EHEC O157 strains was also present in *ureD* of strain Ro8. The upstream region and transcriptional termination signals

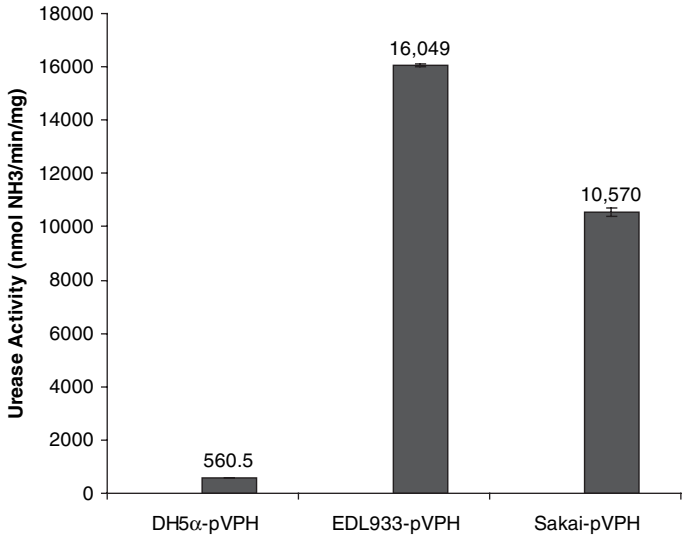

Figure 1. Urease activities of cloned O149 *ure* operon in O157:H7 strains. Each bar represents the average of triplicate assays for urease activity. Error bars indicated standard deviations.

as far as 20 018 bp upstream of the urease operon were also highly conserved (data not shown). Comparison of the translated sequences revealed that the predicted products of the urease operon in O149 strain Ro8 were 99% similar to those of the O157:H7 strains EDL933 and Sakai.

To investigate the functionality of the O149 strain Ro8 urease operon in a number of genetic backgrounds, the entire urease operon with its putative promoter region (∼300 bp upstream of the likely translational start site) was cloned into pGEM-T Easy (a high-copy vector) and pEXT22 (a low-copy vector) and transformed into *E. coli* DH5α and O157:H7 strains, EDL933 and Sakai. All transformants showed no delay in their ability to turn urea agar red and the cloned O149 urease operon resulted in considerable urease activity in the O157:H7 strains EDL933 and Sakai (Fig. 1), indicating that the O149 urease operon is not repressed in these strains.

3.4. Urease deletion mutant

An in-frame nonpolar deletion mutation of the urease operon was constructed in ETEC O149 strain Ro8N, as described above. Ro8NΔ*ureC* had no detectable urease activity on Christensen's agar and in the phenol-hypochlorite urease assay. The urease expression in the Ro8 mutant was restored by complementation with the urease operon from the parent strain cloned in a multicopy plasmid vector (Ro8M-pVPH, Fig. 2).

3.5. Transposon mutagenesis and colony selection

A total of 6 000 transconjugants from the Tn*10* mutant library were screened on Christensen's urea agar plates (Kan/Nal), and on MUM (Kan/Nal) for the identification of urease hyperproducer and urease-negative mutants, respectively. All of the mutant colonies were checked by Southern blotting and shown to possess only a single copy of Tn*10*. The urease-negative mutants were examined by PCR analysis and only those mutants in which the transposon was not inserted in the urease operon were selected for further study. With these strains, inverse PCR and sequencing were then used to determine the location of the Tn*10* transposon. Two insertion sites were identified in urease-negative mutants. In three of the

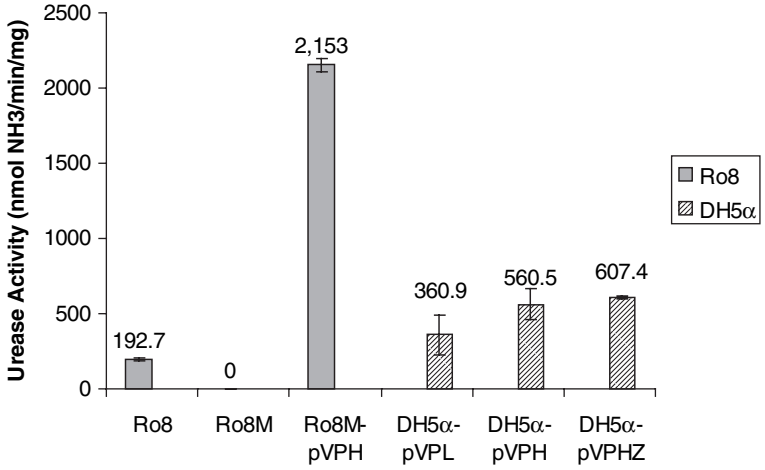

Figure 2. Urease activities of wt-O149 and of cloned O149 *ure* operon in Ro8 and DH5α backgrounds. Each bar represents the average of triplicate assays for urease activity. Error bars indicated standard deviations.

urease-negative mutants the transposon was inserted in the *lipB* gene and in another, the insertion was in the *guaA* gene.

The hyperproducer mutants, named UH6 and UH17, had the Tn*10* insertion in genes *caiF* and Z1580, respectively. The *caiF* gene encodes the transcriptional regulator of the *cai* operon that encodes carnitine dehydratase (CaiB) and crotonobetaine reductase (CaiA), enzymes which are involved in the two-step carnitine pathway [1]. The Z1580 gene, located 375 nucleotides upstream of the urease operon, encodes a putative 93-amino-acid protein of unknown function and is divergently transcribed from the urease operon.

3.6. Urease activity determination by the phenol-hypochlorite urease assay

Urease activity of O149 ETEC and *E. coli* recombinants is shown in Figures 1 and 2. Urease activity expressed from the high copy plasmid in the Ro8 *ureC* mutant strain was approximately 11-fold higher than in the wild type. In the DH5α background, the urease activity was approximately three-fold higher than the Ro8 wild type level. When gene Z1580 immediately upstream of the urease gene cluster was included in the cloned DNA fragment, there was no statistically significant difference in urease expression (Fig. 2). Comparison of the results for the DH5α strains with the low and high copy number plasmids (Fig. 2) suggests that there was not a large gene copy effect in this background.

Surprisingly, when pVPH was transformed into two O157:H7 strains, urease activity was approximately 55- to 83-fold higher compared with the O149 strain Ro8 (Figs. 1 and 2).

3.7. Sequence analysis of the O149 genomic island

The high homology between genes of the urease cluster in the O149 and O157:H7 strains of *E. coli* led us to explore the possibility that a homolog of the urease-containing GI of O157 EHEC may be present in O149:H10 ETEC. PCR amplification demonstrated that O149 strain Ro8 had gene sequences for TerA, Iha, and AIDA-I and an insertion in the *serX* tRNA gene, and that the site of insertion of the putative GI was the same as that of OI-48 of O157:H7 strain EDL933. Long-range PCR demonstrated that there was a continuous fragment of O149 DNA that was not present in *E. coli* K12 and was similar to OI-48 of O157:H7 strain EDL933. At the left junction of this fragment there were sequences that correspond to the first 13 bp of *serX* of *E. coli* K12, followed by sequence present in O157:H7 strain EDL933. At the right junction,

Table III. Markers of OI-48_{149} in strains of porcine O149:H10 and O149:H43 ETEC.

Strains	*serX*	*ureC*	*terA*	*iha*	*aid*	LJ	RJ
15 recent O149:H10	–	+	+	+	+	+	+
1 recent O149:H10	+	+	+	+	+	+	+
1 less recent O149:H10	+	–	–	–	–	–	–
7 recent O149:H43	+	–	–	–	–	–	–
1 recent O149:H43	+	–	–	+	+	–	–
1 recent O149:H43	+	–	+	–	–	–	–
14 less recent O149:H43	+	–	–	–	–	–	–
1 less recent O149:H43	+	–	+	–	–	–	–
1 less recent O149:NM	+	–	–	–	–	–	–
EDL933	-	+	+	+	+	+	+
DH5α	+	–	–	–	–	–	–
1 non-O149 ETEC	–	–	+	+	+	+	+
1 non-O149 ETEC*	+	–	+	+	+	+	+
5 non-O149 ETEC	+	–	–	–	–	–	–

LJ: Left junction of the GI. RJ: Right junction of the GI. –: Absent. +: Present.

there were sequences present in O157:H7 followed by the first nucleotide of the *serX* tRNA gene. All of the PCR amplicons had 97–99% nucleotide sequence similarity to corresponding sequences in *E. coli* O157:H7, indicating that the O149:H10 porcine ETEC strain Ro8 has a GI that is homologous to OI-48 of *E. coli* O157:H7. This OI was designated OI-48_{149}.

3.8. Presence of OI-48_{149} in O149 strains

The primers that were used to identify the left and right junctions of OI-48_{149}, the genes for UreC, TerA, Iha, AIDA, and primers that identify the presence of an intact *serX* tRNA gene were used in PCR reactions with genomic templates from a representative subset of the 130 O149 strains and the seven non-O149 ETEC strains (Tab. III). A total of 49 strains (16 urease-positive and one urease-negative strains of serotype O149:H10, 24 urease-negative strains of serotype O149:H43, 1 urease-negative strain of serotype O149:NM, and 7 non-O149 ETEC strains) were examined. Among O149:H10 ETEC, 15/17 strains had PCR amplification patterns that were identical to that of the positive control *E. coli* strain, EDL933, one was positive for *serX* tRNA gene as well as the other sites tested, and one was typical except that it was negative for *ureC* (Tab. III). All 24 O149:H43 and the one O149:NM isolates lacked the GI. Two of the non-O149 ETEC appeared to possess urease-negative variants of the GI.

3.9. Tellurite resistance test

Tellurite resistance (another marker of the GI) in O149 ETEC was also evaluated. All *terA*-positive strains that were tested (namely, 16 of 17 recent O149:H10 strains, 1 of 9 less recent O149:H43 strains, and 1 of 16 less recent O149:H43 strains) were tellurite resistant (Tab. III). All of the *terA*-negative strains including the negative control strain DH5α, failed to grow on the lowest level of potassium tellurite (1 μg/mL). The MIC of potassium tellurite for the resistant O149:H10 strains and the one less recent O149:H43 strain was 64 μg/mL, the same value for the positive control O157:H7 Sakai strain. The MIC for the one *terA*-positive recent O149:H43 strain (Tab. III) was 256 μg/mL.

4. DISCUSSION

The urease operon and its upstream promoter region in the porcine ETEC strain Ro8 was highly homologous (99% identity) at the nucleotide sequence level with the operons found in O157 EHEC strains EDL933 and Sakai. Despite this similarity there was delayed urease expression in the ETEC

strain whereas no urease expression could be detected in the two O157 EHEC strains. The lack of in vitro expression of the urease operon in O157:H7 strains humans is well established [11, 17, 35]. The delayed appearance of urease activity by O149 ETEC strain Ro8 on Christensen's urea agar is likely due to a low level of urease activity. Compared to O149 ETEC wild type levels, the extent of urease activity following cloning of the genes in a high copy plasmid increased 10-fold in the ETEC strain, 83-fold in O157 EHEC strain EDL933 and 55-fold in O157 EHEC strain Sakai. These are likely due to a gene dosage effect. However, regulation appears to be more complex as there was hardly any increase over wild-type ETEC urease levels when the host bacterium was DH5α. These findings suggest that various *E. coli* strains have different capacities for expression of the urease genes, likely due to different regulation.

Heimer et al. [17] found that when the cloned *ure* operon from the O157 strain EDL933 was transformed into strain EDL933, there was no urease activity, whereas in the present study, when the cloned *ure* operon from O149 ETEC strain Ro8 was transferred into strain EDL933, there was high expression of urease activity (Fig. 1). These observations indicate a distinct difference in regulation of the O149 urease operon compared with the EDL933 urease operon in strain EDL933. Heimer et al. [17] also examined two other O157 EHEC strains in which they detected low levels of urease activity during stationary phase culture in L broth. It would be interesting to see whether these strains possess urease operons that behave like the O149 ETEC urease operon.

Although Nakano et al. [28] showed that the urease operon was non-functional in the Sakai strain of O157:H7 and attributed this to the presence of a stop codon in *ureD*, this is evidently not the case for the operons in EDL933 or strain Ro8, in which the *ure* operons have been demonstrated to be functional. Furthermore, Friedrich et al. [10] have shown that several EHEC strains that lack this stop codon still fail to express urease activity in vitro.

The PCR data showed that the *ure* operon of O149 ETEC strain Ro8 is part of a GI that is highly related to OI-48 of O157 EHEC strain EDL933. This GI includes potential virulence genes such as the urease genes that may be involved in acid resistance [17] and the genes for Iha and AIDA-I that may play a role in adherence [29, 40]. The AIDA-I adhesin has been associated primarily with porcine ETEC and Stx2e strains that carry the F18 fimbriae [16, 29], but has also been reported in F4-positive O149 ETEC [37]. Disease due to ETEC in pigs is quite different from that due to O157 EHEC in humans and involves different areas of the intestine. However, urease in EHEC may be expressed in vivo and acid-resistance mediated by urease may contribute to virulence in both types of disease. Urease genes have been detected in serogroups of EHEC such as O157, O26, O111 and O145 that are associated with high virulence [11, 27] and these genes are associated with highly virulent O149 porcine ETEC [31]. It is clear, however, that both ETEC and EHEC can be highly virulent despite not having urease genes, suggesting that the contribution of urease to virulence is likely a small one.

Among the O149 ETEC strains, all the marker genes and both junctions for the OI were detected in the 16 recent O149:H10 strains that were examined (Tab. III). In contrast, the less recent O149 strains all lacked the markers and junctions associated with the GI, although one or two of the genes present on the GI were detected in some of these strains. Interestingly, two of the three non-O149 porcine ETEC that were investigated appear to have the GI, but no *ureC* gene. It may be worthwhile to investigate the presence of this GI in a large collection of porcine ETEC of a range of serotypes.

Four genes were identified as having a role in expression of urease activity. The *lipB* gene encodes the lipoyl(octanoyl)transferase [20] and has a role in the regulation of *dam*-encoded deoxyadenosine methylase, which in turn regulates a large variety of key cell processes [43], but its likely relationship to the *ure* genes is not evident. Since three mutants

showed the presence of the Tn*10* tranposon in the *lipB* gene, this gene appears to possess a high-affinity site for transposition. The *guaA* gene encodes GMP synthetase and is needed for synthesis of GTP, which is required for the function of the urease accessory genes that transport urea into the bacterial cell [38].

The hyperproducing mutants UH6 and UH17 had the Tn*10* insertion in genes *caiF* and Z1580, respectively. Gene *caiF* encodes the transcriptional regulator of the *cai* operon that is responsible for anaerobic metabolism of dietary carnitine in the intestine [1]. Gene Z1580 is located 375 nucleotides upstream of the urease operon and encodes a putative 93-amino-acid protein of unknown function transcribed in the opposite direction to the urease operon. The links between these genes and urease are not known.

The following conclusions are drawn from this study. Strains of O149:H10 porcine ETEC typically possess a urease operon which is highly related at the nucleotide sequence level to the urease operon in O157 EHEC strain EDL933. The O149 urease operon results in a delayed urease positive phenotype on Christensen's urea agar, which is likely due to a low level of urease activity. The O149 urease operon is a part of a GI similar to OI-48 in O157 EHEC strain EDL933. Both islands possess genes for urease, tellurite resistance, and adhesins Iha and IIDA-1 and are inserted into the *serX* site in the chromosome. The level of expression of the O149 urease operon varies with the strain in which it is present. The levels of expression of the cloned urease genes in a multicopy plasmid were 42–65 times higher in two strains of O157 EHEC compared with the *E. coli* K12 strain DH5α.

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