International Journal of Medical Microbiology 309 (2019) 108–115

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

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

Phenotypic and molecular characterization of *Escherichia albertii*: Further surrogates to avoid potential laboratory misidentification

Atsushi Hinenoya[a,b,1], Hidetoshi Ichimura[a,1], Sharda Prasad Awasthi[b], Noritomo Yasuda[b], Jun Yatsuyanagi[c,2], Shinji Yamasaki[a,b,*]

[a] School of Life and Environmental Sciences, Osaka Prefecture University, Osaka, Japan
[b] Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Osaka, Japan
[c] Akita Research Center for Public Health and Environment, Akita, Japan

ARTICLE INFO

Keywords:
Escherichia albertii
eae
E. coli cytolethal distending toxin-II
CDT
EPEC

ABSTRACT

Escherichia albertii is an emerging gastrointestinal pathogen, related to *Escherichia coli*, which can be misidentified as enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC), due to the presence of the *eae* gene in *E. albertii*. The aim of this study was to verify our hypothesis that *E. coli* cytolethal distending toxin-II (*Eccdt-II*) gene-positive *E. coli* is *E. albertii* and to accumulate the data regarding the bacteriological characteristics of *E. albertii*. For these purposes, we attempted to detect *E. albertii* in *eae* gene-positive bacteria previously identified as *E. coli* and to examine if re-identified *E. albertii* contained *Eccdt-II*-homologous gene and remaining *eae* gene-positive *E. coli* did not. A total of 373 *eae* gene-positive *E. coli* strains were analyzed by biochemical tests, multilocus sequence analysis and an *E. albertii*-specific PCR. The strains re-identified as *E. albertii* were also examined for the presence of *cdt* genes by using ^{32}P-labled DNA probes, followed by their toxin-typing. Of the 373 strains, 17 were re-identified as *E. albertii* by three above-mentioned methods. Furthermore, all the 17 re-identified *E. albertii* possessed *cdt* genes highly homologous to *Eccdt-II* and *Eacdt* genes. Moreover, *Eccdt-I* or both *Eccdt-I* and *stx2f* genes were detected in two re-identified *E. albertii* strains. However, the remaining 356 strains did not carry such *cdt* genes. These data indicate that all re-identified *E. albertii* isolates specifically carried *cdt* genes homologous to *Eccdt-II* and *Eacdt* genes. We suggest that *Eccdt-II* gene-positive *E. coli* may be identical to *E. albertii*.

1. Introduction

Escherichia albertii is a recently recognized human enteropathogen and avian pathogen (Huys et al., 2003; Oaks et al., 2010). This bacterium was first isolated from a 9-month-old girl with diarrhea in Bangladesh and originally described as *Hafnia alvei* by routine bacterial identification tests based on their biochemical properties (Albert et al., 1991, 1992). Thereafter, extensive characterization of these *Hafnia alvei* strains allowed for the reclassification of these isolates to be a member of the genus *Escherichia*, and the new taxon, *E. albertii*, was therefore proposed. (Huys et al., 2003).

E. albertii joins six other species within the genus *Escherichia*, including *Escherichia adecarboxylata* (Leclerc, 1962), *Escherichia coli* (Migula, 1895), *Escherichia fergusonii* (Farmer et al., 1985), *Escherichia hermannii* (Brenner et al., 1982a), *Escherichia marmotae* (Liu et al., 2015), *Escherichia vulneris* (Brenner et al., 1982b). *Escherichia blattae* has been recently reclassified as *Shimwella blattae* (Priest and Barker, 2010).

E. albertii is one of the attaching and effacing pathogens, where the *eae* gene encoding intimin, an outer membrane protein associated with binding to intestinal epithelium, is commonly present. In addition, *E. albertii* produce cytolethal distending toxin (CDT) consisting of 3 different subunits, CdtA, CdtB and CdtC. Although it has been reported that various Gram-negative bacteria such as *Escherichia coli* producing 5 different CDTs (EcCDT-I through EcCDT-V), *Campylobacter* spp, and *Shigella* spp. produce species-specific CDT (Yamasaki et al., 2006), the clinical significance of the function of CDT remains unclear. CDT is, however, considered to be associated with increased persistent

* Corresponding author at: Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-58, Rinku ourai-kita, Izumisano, Osaka, 598-8531, Japan.
E-mail address: shinji@vet.osakafu-u.ac.jp (S. Yamasaki).
[1] These authors contributed equally to this work.
[2] Present address: Yokote Institute of Public Health, Akita, Japan.

https://doi.org/10.1016/j.ijmm.2018.12.003
Received 25 October 2018; Received in revised form 16 December 2018; Accepted 28 December 2018

colonization and invasion of the bacteria, as well as with severity of the disease (Fox et al., 2004; Ge et al., 2005, 2007; McAuley et al., 2007; Pandey et al., 2003; Young et al., 2004). Furthermore, certain strains of *E. albertii* have also been reported to produce Shiga toxin 2 (Stx2a, Stx2f) or carry those genes (Brandal et al., 2005; Hinenoya et al., 2017a; Murakami et al., 2014; Ooka et al., 2012), which are primary virulence factors of enterohemorrhagic *E. coli* (EHEC), including serotype O157:H7.

E. albertii is difficult to differentiate from *E. coli* solely by routine biochemical tests. Indeed, in addition to *H. alvei*, *E. albertii* were previously misidentified on occasions as *E. coli* such as enteropathogenic *E. coli* (EPEC) and EHEC (Hinenoya et al., 2009; Oaks et al., 2010; Ooka et al., 2012), as the *eae* gene is commonly present in both EPEC and EHEC. In addition to *eae* gene, EPEC carry bundle forming pili (*bfp*) gene, while EHEC harbor *stx1* and/or *stx2* genes. In *stx2* genes, there are at least 7 subtypes (*stx2a-stx2g*) so far. Recently, Centers for Disease Control and Prevention reported 'National Enteric Disease Surveillance: *Shigella* Surveillance Overview' in 2011 in which *Shigella boydii* serotype 13 is regarded as *E. albertii*. This report was based on the 2 publications showing results of DNA-DNA hybridization analysis of *E. coli* and *Shigella* spp. including the serotype 13 (Brenner et al., 1982c), and evolutional relatedness of *E. albertii* with *S. boydii* strains (Hyma et al., 2005), respectively. These reports suggest that a large proportion of *E. albertii* could be misidentified as other bacterial species, leading to underestimation of *E. albertii*-related diseases and paucity of available data about reliable bacterial characteristics of *E. albertii*.

Our recent studies regarding re-identification of *Eccdt-II* gene-positive *E. coli* as *E. albertii* (Hinenoya et al., 2014, 2017a, 2017b) suggested that *Eccdt-II* gene-positive *E. coli* is actually *E. albertii* but not *E. coli*. However, since these findings were demonstrated from *Eccdt-II* gene-positive *E. coli* to *E. albertii*, demonstration from opposite side is also essential. Namely, it should be proved that if *E. albertii* re-identified from *eae* gene-positive *E. coli* carry *cdt* genes homologous to *Eccdt-II* genes, and the remaining *eae* gene-positive *E. coli* does not carry the *cdt* genes.

It was therefore the aim of this study, to retrospectively re-examine a large collection of gastrointestinal pathogens and environmental isolates, originally identified as *eae* gene-positive *E. coli*, specifically checking for the presence of *E. albertii* through employment of biochemical tests, multilocus sequence (MLS)-analysis and an *E. albertii*-specific PCR. Furthermore, re-identified *E. albertii* were subjected to bacteriological characterization, especially to see if *cdt* genes homologous to *Eccdt-II* genes are ubiquitously present.

2. Materials and methods

2.1. Bacterial strains

A total of 373 *eae* gene-positive *E. coli* isolates were examined in this study, which were originally isolated from humans, animals, foods and environment between 1991 and 2002, and between 2008 and 2012 in Akita prefecture, Japan. Detailed information of the isolates used in this study is summarized in Table S1. Each isolate was streaked on a LB agar plate (Difco™ LB Broth, Lennox; Becton, Dickinson and Company, Franklin Lakes, NJ, USA, Agar Powder; Nacalai Tesque, Inc., Kyoto, Japan) from glycerol stocks kept at −80 °C, and incubated at 37 °C overnight. Resulting pure single colonies were subjected to the following experiments.

2.2. Biochemical properties and motility

Biochemical properties and motility were examined by conventional methods (Ewing, 1986) using TSI (triple sugar iron) slants and SIM (sulfide indole motility) (Eiken Chemical Co., Ltd., Tokyo, Japan). Furthermore, acid production from carbohydrates was examined for 48 h incubation at 37 °C under an aerobic condition by using OF basal medium (Eiken Chemical Co., Ltd.) containing one of the following 9 carbohydrates at the concentration of 1% w/v: dulcitol, α-D-(+)-melibiose, L-(+)-rhamnose, L-sorbitol, sucrose, D-(+)-xylose (Nacalai Tesque, Inc.), lactose monohydrate (Kanto Chemical Co., Inc., Tokyo, Japan), maltose monohydrate, and D-(-)-mannitol (Wako Pure Chemical Industries, Ltd., Osaka, Japan), respectively. Motility was examined by 24 h incubation at 37 °C in SIM medium (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan).

2.3. Preparation of template DNA solution

Bacterial strains were inoculated into 3 mL of LB broth (Becton, Dickinson and Company) and incubated at 37 °C overnight under an aerobic condition with vigorous shaking. An aliquot of each bacterial culture was diluted 10 times with TE-buffer (10 mM Tris − HCl, 1 mM EDTA [pH 8.0]), boiled for 10 min and centrifuged at 15,000 x *g* for 3 min at 4 °C. Resulting supernatant was collected and used as a template DNA in the following experiments.

2.4. Multilocus sequence (MLS) analysis

MLS analysis was performed according to the protocol of University of Warwick with slight modifications (Wirth et al., 2006). Briefly, 7 housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *purA*, *mdh*, and *recA*) were PCR amplified using TaKaRa Taq™ (Takara Bio Inc., Shiga, Japan) and sequenced by a cycle sequencing method using BigDye® Terminator v1.1 and ABI 3130 Genetic Analyzer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The primers and PCR conditions are shown in Table S2. The loci were concatenated into a 3,423 bp sequence in the order mentioned above. The phylogenic tree was constructed by the maximum likelihood model using MEGA6 software with default setting and boot-strapping 500 times (Tamura et al., 2013). The strains shown in Table S3 were included into MLS analysis as reference.

2.5. Virulence gene profiling and determination of LEE integration site

Presence of *eae*, *stx1*, *stx2, cdt*, and *bfp* genes was analyzed by colony hybridization assay using ^{32}P-labelled gene-probes, including *eae*, *stx1*, *stx2a*, *Eccdt-I* and *Eacdt*, and *bfp*, respectively, under high stringent conditions, as previously described (Yamasaki et al., 1996). DNA probes were prepared as previously described (Hinenoya et al., 2009). Briefly, partial sequences of *eae*, *stx1*, *stx2a* and *stx2f*, *Eccdt-IB* and *EacdtB*, and *bfp* genes were amplified by PCRs using primer sets of EccdtB-comU and EccdtB-comD, EcCDTII-F2 and EcCDTII-R2, EVT-1 and EVT-2, EVS-1 and EVC-2, 128-1 and 128-2, and bfp-F and bfp-R, respectively (Table S2). EcCDT-I-producing *E. coli* strain GB1371 (Pandey et al., 2003), and *E. albertii* strain P2660 (Hinenoya et al., 2017a) were used as a template DNA for *eae, Eccdt-IB* and *bfp* genes, and *Eacdt-IIB* and *stx2f* genes, respectively. *E. coli* O157:H7 strain Sakai was used as a template DNA for *stx1* and *stx2a* genes. *In silico* analysis indicates that the *EacdtB* and *stx2f* gene-probes can detect all the corresponding genes of *E. albertii* so far registered in the GenBank at NCBI and DNA Data Bank of Japan (DDBJ). PCR products were gel-cut purified by using Wizard® SV Gel and PCR Clean-Up System (Promega, Fitchburg, WI, USA), and labelled with ^{32}P by a random priming method using Megaprime DNA Labelling System (GE Healthcare Life Sciences, Buckinghamshire, UK) and [α-^{32}P]-dCTP (370 MBq/mL) (PerkinElmer Inc., Waltham, MA, USA). When *cdt* and *stx2* genes were detected by colony hybridization assay, a PCR-RFLP assay, which can differentiate *Eccdt-I*, *Eccdt-II* (*Eacdt*), *Eccdt-III/Eccdt-V* and *Eccdt-IV*, *Eccdt-III-* and *Eccdt-V*-specific PCRs to distinguish them (Hinenoya et al., 2009; 2014), and subtype-specific PCRs for *stx2* genes (Beutin et al., 2007) were performed to determine *cdt* and *stx2* subtypes, respectively.

The LEE integration site was determined by PCR described by Ooka et al. (2012) with slight modifications. Briefly, genomic DNA was isolated from enrichment culture as described elsewhere (Wilson, 2001).

Table 1
Biochemical properties, antimicrobial resistances and genetic profiles of re-identified *Escherichia albertii* strains.

Strain	Motility[*1]	Utilization of carbohydrates (1% w/v)[*2]							*E. albertii* PCR[*3]	Antimicrobial resistance[*4]	Virulence gene properties	CDT titer[*5]	Intimin subtype	LEE integration site
		Dul	Lac	Mel	Rha	Sor	Suc	Xyl						
AKT5	-[*6]	-[*7]	–	–	–	+[*8]	–	–	+[*9]	NR[*10]	*eae*, *Eccdt-I*, t*Eacdt*[*11], *stx2f*	16	unknown (N2)	*pheU*
AKT11	–	–	–	–	–	+	+	–	+	NR	*eae*, *Eacdt*	16	unknown (N1.2)	*pheU*
AKT22	–	–	–	–	–	+	–	–	+	NR	*eae*, *Eacdt*	16	beta3	*pheU*
AKT72	–	–	–	–	–	+	–	–	+	NR	*eae*, *Eacdt*	8	beta3	*pheU*
AKT73	–	–	–	–	–	–	–	–	+	NR	*eae*, *Eacdt*	8	unknown (N3)	ND[*12]
AKT80	–	–	–	–	–	+	–	–	+	NR	*eae*, *Eacdt*	8	sigma	*pheU*
AKT92	–	–	–	–	–	+	+	–	+	NR	*eae*, *Eacdt*	8	unknown (N2)	*pheU*
AKT109	–	–	–	–	–	+	–	–	+	NR	*eae*, *Eacdt*	8	beta3	*pheU*
AKT123	–	–	–	–	–	+	–	–	+	NR	*eae*, *Eacdt*	16	unknown	*pheU*
AKT128	–	–	–	–	–	+	–	–	+	NR	*eae*, *Eacdt*	32	beta3	*pheU*
AKT130	–	–	–	–	–	–	–	–	+	ABPC	*eae*, *Eccdt-I*, *Eacdt*	8	omicron	*pheU*
AKT131	–	–	–	–	–	–	–	–	+	NR	*eae*, *Eacdt*	8	sigma	*pheU*
AKT148	–	–	–	–	–	–	–	–	+	NR	*eae*, *Eacdt*	4	sigma	*pheU*
AKT152	–	–	–	–	–	–	–	–	+	NR	*eae*, *Eacdt*	8	xi	*pheU*
AKT219	–	–	–	–	–	+	–	–	+	NR	*eae*, *Eacdt*	8	unknown	*pheU*
AKT265	–	–	–	–	–	–	–	–	+	NR	*eae*, *Eacdt*	4	iota2	ND
AKT294	–	–	–	–	–	+	–	–	+	NR	*eae*, *Eacdt*	8	beta1	*pheU*

[*1]motility in SIM medium [*2]Dul, dulcitol; Lac, lactose; Mel, α-D-(+)-melibiose; Rha, L-(+)-rhamnose; Sor, L-sorbitol; Suc, sucrose; Xyl, D-(+)-xylose [*3]developed by Ooka et al. (2015) [*4]ampicillin (ABPC), ceftazidime, cefoxitin, chloramphenicol, ciprofloxacin, cefotaxime, fosfomycin, gentamicin, imipenem, kanamycin, nalidixic acid, norfloxacin, streptomycin, trimethoprim-sulfamethoxazole and tetracycline were tested. [*5]Reciprocal of the highest dilution of filtrated cell sonic lysates of TSB culture that resulted in cytoplasmic distension in > 50% of cells [*6]negative for motility [*7]negative for utilization of carbohydrates [*8]positive for utilization of carbohydrates [*9]Expected size of PCR product was yielded. [*10]no resistance to any of antimicrobials tested [*11]truncatated *Eacdt* genes [*12]not determined.

The genomic DNA was subjected to PCRs using TaKaRa LA Taq™ (Takara Bio Inc.), escR primer, a common reverse primer and one of the forward primers (pheV-Ro1, pheV-glcB, selC-Ro1 and 433-f). PCR primer sequences and a PCR condition are shown in Table S2.

2.6. Sequencing of entire cdt, eae and stx2f genes

The entire nucleotide sequences of *Eccdt-I*, *Eacdt*, *stx2f*, and *eae* genes were determined as previously described (Hinenoya et al., 2017a). A neighbor-joining tree based on nucleotide sequences of the *cdt* genes was constructed by MEGA6 software. To determine intimin subtypes, predicted amino acid sequences of *eae* genes were aligned with those of the reference intimin subtypes by the Clustal W program of MEGA6. The reference intimin subtypes used were from Ooka et al. (2012). Nucleotide and predicted amino acid sequences of *cdt* genes and intimin of the strains were listed in Table S3 and included as references.

2.7. Antimicrobial susceptibility

Re-identified *E. albertii* strains were analyzed for antimicrobial susceptibility by disk diffusion method following the protocol of CLSI M07-A11. Zone of inhibition size was interpreted based on the criteria of the CLSI M100-S26 for the family *Enterobaceriaceae*. The following 15 antimicrobial agents including cefotaxime (30 μg), ceftadizime (30 μg), cefoxitin (30 μg), imipenem (30 μg), ampicillin (10 μg), fosfomycin (50 μg), streptomycin (10 μg), kanamycin (30 μg), gentamicin (10 μg), chloramphenicol (30 μg), tetracycline (30 μg), norfloxacin (10 μg), ciprofloxacin (5 μg), nalidixic acid (30 μg), and sulfamethoxazole-trimethoprim (1.25 μg/23.75 μg) were used (Becton, Dickinson and Company).

2.8. Cytotoxicity assay

Cytotoxicity assay was performed using Vero and CHO cells, respectively, as described previously (Hinenoya et al., 2017a) with slight modifications. *E. albertii* strains were cultured in 3 mL of LB-broth at 37 °C overnight. An aliquot of the overnight culture was inoculated into 3 mL of fresh tryptic soy broth or LB-broth and cultured to early log phase, followed by further aerobic incubation for 6 h with or without 0.5 μg/mL of mitomycin C (MMC; Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan). Culture supernatant or cell sonic lysate was passed through a sterile filter with 0.2-μm pore size (Asahi glass Co., Ltd., Tokyo, Japan), and the filtrate was used for cytotoxicity assay.

To neutralize the activities of EcCDT-I, EaCDT and/or Stx2f, the filtrate diluted appropriately was incubated at 37 °C with equal volume of anti-EcCdt-IB, anti-PaCdtC and/or anti-Stx2fA sera for 30 min. The mixtures were applied to the cytotoxicity assay. Anti-PaCdtC serum could also neutralize EaCDT activity due to high amino acid sequence homology between EaCdtC and PaCdtC (> 86.8%) as previously described (Hinenoya et al., 2017a).

2.9. Nucleotide sequence accession numbers

All nucleotide sequences obtained in this study were deposited into the DDBJ database. The accession numbers are LC415145-LC415263 (for the 7 housekeeping genes [*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, *recA*] used for MLS analysis), LC415264-LC415282 (for the *cdt* genes), LC415283-LC415299 (for the *eae* genes) and LC415300 (for the *stx2f* genes), respectively.

3. Results

3.1. Detection of E. albertii by phenotypic characterization of eae gene-positive E. coli

A total of 373 *eae* gene-positive *E. coli* strains isolated from symptomatic and asymptomatic humans, foods and environment were analyzed for their phenotypic properties to detect *E. albertii* (Tables 1 and S1). Among these, 17 strains shown in Table 1 could not utilize any of the 4 carbohydrates including dulcitol, α-D-(+)-melibiose, L-(+)-rhamnose and D-(+)-xylose, which are utilized by most of *E. coli* but not *E. albertii* isolates reported to date (Abbott et al., 2003;

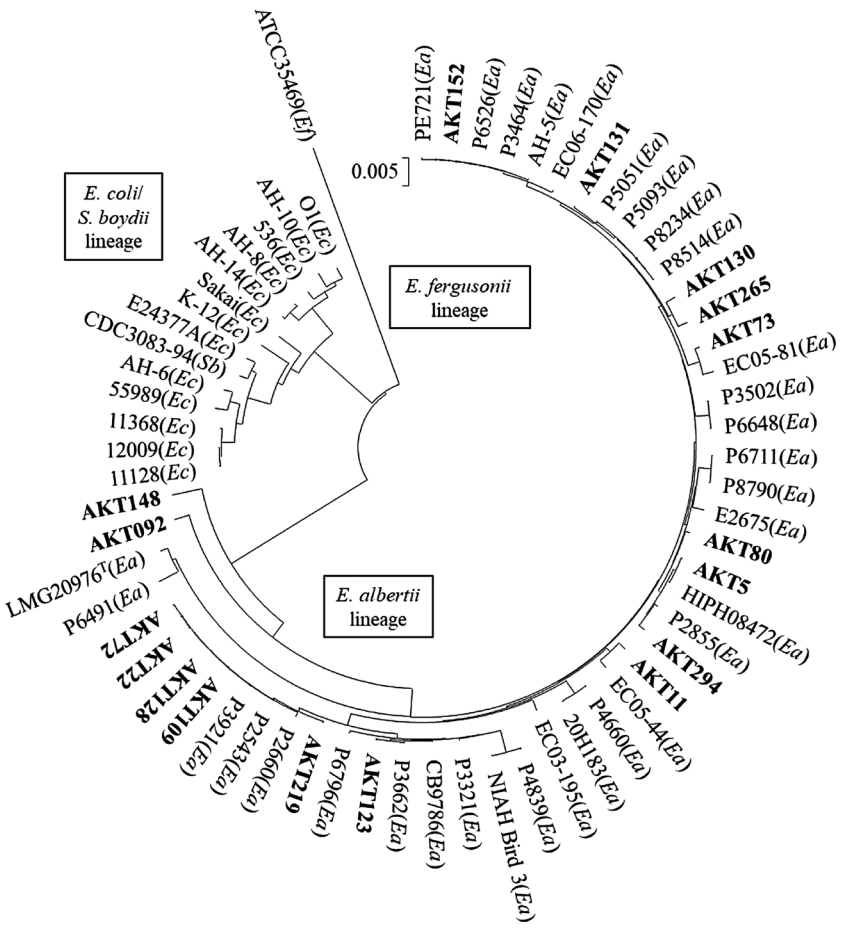

Fig. 1. Neighbor-joining tree of *E. albertii* candidates by multilocus sequence analysis.

Partial sequences of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) in 17 *E. albertii* candidates detected by biochemical characterizations (highlighted by bold letters) were determined and concatenated in the order described above. The dendrogram was constructed with the concatenated sequences. In addition to 26 *E. albertii* (*Ea*) strains, 13 *E. coli* (*Ec*) strains, 1 *S. boydii* (*Sb*) strain and 1 *E. fergusonii* (*Ef*) strain listed in Table S3 were included as references. Bacterial species of reference strains (*Ec*, *Ea*, *Sb*, *Ef*) are indicated in brackets. Scale bars indicate genetic distance.

Hinenoya et al., 2014, 2017a, 2017b; Oaks et al., 2010; Ooka et al., 2012), and were non-motile, which is a typical characteristic of *E. albertii* under the experimental condition. The remaining 356 strains could utilize more than 2 of the carbohydrates regardless of their motility. Therefore, the 17 strains were selected as *E. albertii* candidates for further studies.

3.2. Confirmation of *E. albertii* candidates by genetic methods

To confirm the species of the 17 *eae* gene-positive presumptive *E. albertii*, MLS analysis which can clearly differentiate *E. albertii* from *E. coli*, and an *E. albertii*-specific PCR previously reported (Ooka et al., 2015) were performed. All the 17 strains were placed in an *E. albertii* cluster distinct from those of *E. coli*, *S. boydii* and *E. fergusonii* on the phylogenic tree (Fig. 1), and produced expected size of amplicons by the PCR (Table 1). These data revealed that these 17 *eae* gene-positive *E. coli* were *E. albertii* but not *E. coli* as previously characterized (Hinenoya et al., 2017b). Among 17 re-identified *E. albertii*, 15, 1 and 1 strains were human isolates from sporadic, epidemic and imported cases, respectively.

3.3. Virulence gene profile and determination of LEE integration site

The 17 strains of *E. albertii* and remaining 356 *eae* gene-positive *E. coli* were also analyzed for the presence of virulence genes including *cdtB* (*Eccdt-IB* and *EacdtB*), *stx* (*stx1* and *stx2*), *bfp*, and *eae* genes by colony hybridization assays. All the strains analyzed in this study were re-confirmed to be *eae* gene-positive. Regarding the 17 *E. albertii* strains, all were positive for *cdtB* gene but not *bfp* gene. The *cdtB* gene was also detected in 2 strains (AKT177 and AKT377) among the 356 *eae* gene-positive *E. coli* (Table S1). Then, *cdt* genes were typed by PCR-RFLP and PCRs specific for *Eccdt-III* and *Eccdt-V*. *cdt* genes in all *E. albertii* strains were typed to be *Eccdt-II*, which is indeed *Eacdt*, but strains AKT5 and AKT130 also contained *Eccdt-I* genes. *cdt* genes in *eae* gene-positive *E. coli* strains AKT177 and AKT377 were typed to be *Eccdt-V*. In case of AKT5, in addition to *Eccdt-I* and *Eacdt-II* genes, *stx2* genes were also detected and further subtyped to be *stx2f* by subtype-specific PCRs (Beutin et al., 2007). *bfp* gene was detected in 30 *eae* gene-positive *E. coli* strains.

Sequence analysis of the *Eacdt* revealed that complete *cdtA* (777 bp), *cdtB* (810 bp) and *cdtC* (546 bp) genes were identified in the 16 strains, except a strain AKT5. As shown in Table S4, the *cdt* genes in 14 and 3 *E. albertii* were highly homologous to *Eccdt-II* genes of strain 9142-88 (98.6–99.6%) and *Eacdt* genes of strain LMG20976^{T} (99.2–99.6%), respectively. *cdt* genes in *E. albertii* belonged to a distinct cluster, which can be divided into two subclusters with the exception of one strain (Bird_8), subcluster 1 for *Eacdt* type strain LMG20976^{T} and subcluster 2 for *Eccdt-II* type strain *E. coli* 9142-88 (Fig. 2). Putative amino acid sequences of *EacdtB* (810 bp) and *EacdtC* (546 bp) in strain AKT-5 were 99.9% (268/269 aa) and 100% (181/181 aa) identical to those of *E. albertii* strain AH-5 (Accession number AB472834). However, the *EacdtA* gene sequence consisted of 778 bp nucleotides, in which a frame shift mutation occurred due to an additional deoxyadenosine nucleotide (A) in the A rich region between nucleotide position 75 and 81 of the *EacdtA*. This result suggests that strain AKT5 cannot produce biologically active EaCDT.

Eccdt-I genes in strain AKT130 were identical to those of *E. coli* strain E6468/62 producing prototype EcCDT-I. On the other hand, 2 nucleotides difference (C919 T, A1242 G) was found in the *Eccdt-IB* gene of strain AKT5 in comparison to those of *E. albertii* strain P2660 (LC140875), however the putative amino acid residues important for its DNase I activity (H153, G190-N193, D228, D259 and H260) were

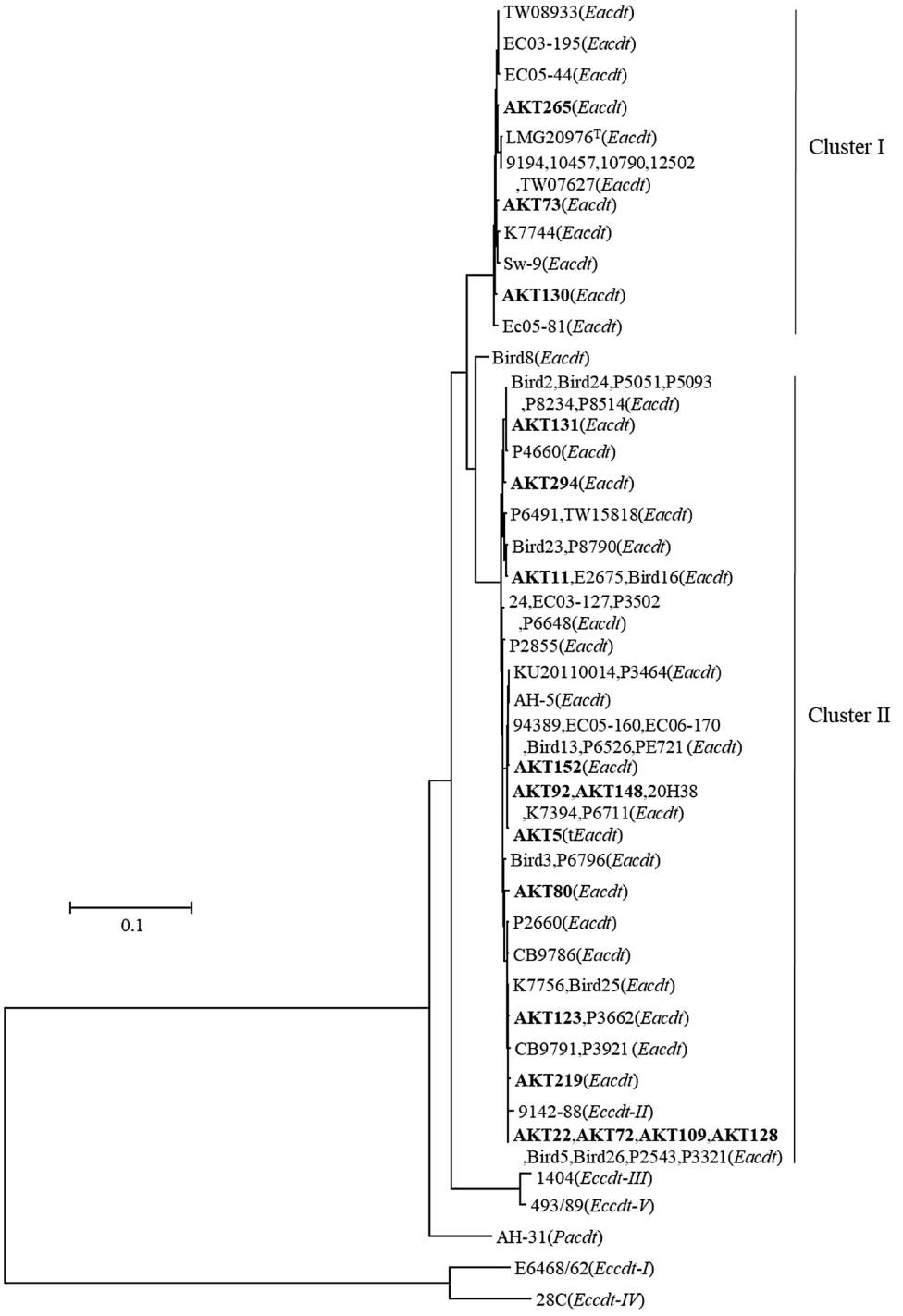

Fig. 2. Neighbor-joining tree-based on nucleotide sequences of *cdt* genes in the *Escherichia albertii* strains analyzed. The dendrogram was constructed with entire *cdt* genes sequences (*Eacdt*, *Eccdt-I*) in 17 *E. albertii* strains used in this study (highlighted by bold letters). The *cdt* genes are designated by their strain names. *cdt* genes from 57 *E. albertii* strains (*Eacdt*), 5 *E. coli* strains (*Eccdt-I*, *Eccdt-II*, *Eccdt-III*, *Eccdt-IV*, *Eccdt-V*), and 1 *P. alcalifaciens* strains (*Pacdt*) listed in Table S3 were included as references. t*Eacdt* indicates truncated *Eacdt*. *Eacdt* and *Eccdt-II* with the exception of strain Bird_8 were comprised of 2 clusters (clusters I and II). Scale bars indicate nucleotide substitutions (%) per site.

conserved. *stx2f* genes identified in strain AKT5 were identical to those of *E. albertii* strain F08/101-31 (AB472687).

Intimin subtypes in the 17 *E. albertii* strains were determined by entire *eae* gene-sequencing and subsequent phylogenic analysis using the putative amino acid sequences (Fig. S1). Intimin subtype was diverse and determined to be beta3 (4 strains), sigma (3), beta1 (1), omicron (1), xi (1) and iota2 (1) whereas 6 strains were unknown subtypes (Table 1). Among the unknown intimin subtypes of the 6 strains, those of 1, 2 and 1 strains were subtyped to N1.2, N2 and N3, respectively, which were tentatively described in the study of Ooka et al. (2012). The remaining 2 were homologous to those of strains P3662 and P3921 described in our previous study (Hinenoya et al., 2017b). In 15 *E. albertii* strains, only *pheU* locus was determined for LEE integration site by PCR using 4 primer sets as described in Table S2. LEE integration site of the remaining 2 strains could not be determined due to no amplicons by any of the 4 primer sets.

3.4. Antimicrobial susceptibility of re-identified E. albertii strains

The 17 re-identified *E. albertii* strains were examined for antimicrobial susceptibility. As shown in Table 1, only strain AKT130 showed ampicillin resistance while the remaining 16 strains did not show any resistance to 15 antimicrobials tested.

3.5. Cytotoxicity and its neutralization assays of E. albertii strains

Production of CDT by re-identified *E. albertii* strains was examined by cytotoxicity assay with CHO cells, which are not sensitive to Stx. The filter sterilized cell sonic lysates of all the re-identified *E. albertii* strains tested induced cell distention at the titer from 4 to 32 after 72 h incubation (Table 1). Morphological changes of the cells were similar to those by *E. albertii* type strain (LMG20976^T) and EcCDT-I-producing *E. coli* strain GB1371 (Data not shown). The CDT activities except for strains AKT5 and AKT130 were neutralized by anti-PaCdtC serum, indicating that the 15 *E. albertii* strains produced biologically active EaCDT. As expected based on the *cdt* gene-sequencing data, the activity of strain AKT5 was neutralized by anti-EcCdtIB serum but not by anti-PaCdtC serum, indicating that strain AKT5 produced functional EcCDT-I but not EaCDT. For strain AKT130, CDT activity was completely neutralized only when both anti-EcCdtIB and anti-PaCdtC sera were present, but not either one of them. This data indicated that strain AKT130 produced functional EcCDT-I as well as EaCDT.

Stx2f production in strain AKT5 was also examined by using Vero cells, which are sensitive to Stx (Fig. S2). Intoxication by the filter sterilized cell sonic lysates caused cell death at the titer of 1,024, as observed in that of *E. albertii* strain P2660 producing Stx2f. The cell death was neutralized by anti-Stx2fA serum, indicating that the strain AKT5 produced biologically active Stx2f.

The titer of CDT activity in the culture supernatant of strain AKT130 was not enhanced when cultured in the presence of MMC (Table 2). However, MMC enhanced its EcCDT-I and Stx2f activities in strain AKT5 by 8- and 32-fold, which were neutralized by anti-EcCdtIB and anti-Stx2fA sera, respectively (Table 2).

4. Discussion

E. albertii isolates possess species-specific *cdt* genes (*Eacdt*), which are located on chromosomal DNA but not mobile genetic elements such as phages, transposons and plasmids (Ooka et al., 2015). These are almost identical to *Eccdt-II* genes in *E. coli* (Hinenoya et al., 2014, 2017a; Ooka et al., 2012). However, our recent studies (Hinenoya et al., 2017b) suggested misidentification of *E. albertii* as *Eccdt-II* gene-positive *E. coli*. Therefore, in order to examine the potential for misidentification, we performed a retrospective analysis of *eae* gene-positive isolates that were originally identified as typical or atypical EPEC by routine biochemical tests. In addition, we examined the occurrence of *cdt* genes, followed by their phylogenetic characterization in all re-assigned *E. albertii* isolates. Overall, we successfully detected 17 (4.6%) isolates as *E. albertii* including one *stx2f* gene-positive. All the 17 re-identified *E. albertii* isolates harbored *cdt* genes highly homologous to *Eccdt-II* and *Eacdt* (93.5–99.6%) which are placed into a cluster distinct from other *cdt* genes. Taken together, although *E. albertii* and *Eccdt-II* gene-positive *E. coli* were regarded as independent bacteria each other, we suggest that *Eccdt-II* gene-positive *E. coli* may be identical to *E. albertii*.

Table 2
Effect of mitomycin C on toxin production by EcCDT-I and Stx2f-producing *Escherichia albertii*.

				Toxin titer[$]	
Species	Strain	Toxin gene	MMC[*]	CHO	Vero
E. albertii	AKT5	*Eccdt-I, tEacdt, stx2f*	–	4	512
			+	32	16,384
	AKT130	*Eccdt-I, Eacdt*	–	32	ND
			+	< 1	ND
	P2660	*Eccdt-I, Eacdt, stx2f*	–	2	8
			+	16	128
E. coli	Sakai	*stx1, stx2*	–	ND[$]	512
			+	ND	32,768
	GB1371	*Eccdt-I*	–	4	ND
			+	32	ND
	C600	–	–	< 1	< 1

[*] mitomycin C, #not done, Filtrated culture supernatants in LB-broth with and without MMC were used as toxin samples.
[$] Reciprocal of highest dilution that resulted in cytoplasmic distension or death in > 50% of cells is shown as toxin titer.

MLS analysis is the sole method, which can clearly differentiate *E. albertii* from other *Enterobacteriaceae* including *E. coli* and *S. boydii*, however it is not suitable for routine bacterial identification tests because it is time-consuming, labor-intensive and not cost-effective. PCRs targeting *E. albertii*-specific nucleotide sequences have been reported (Hyma et al., 2005; Ooka et al., 2015; Lindsey et al., 2017). However, since they were developed based on the genetic properties of limited number of *E. albertii* strains from limited sources, their sensitivity and specificity are still uncertain. Indeed, it was reported that *Klebsiella variicola* was determined as *E. albertii* (Maeda et al., 2014). Therefore, there is still much information left about the properties of *E. albertii* in order to construct simple and reliable methods to detect and identify *E. albertii*. Thus, we have also extensively analyzed the bacteriological properties of the re-identified *E. albertii* strains

In comparison to low variation in LEE integration site of *E. albertii* for which only *pheU* gene was identified, various intimin subtypes including alpha8, beta1, beta3, epsilon1, epsilon3, epsilon4, iota2, nu, omicron, rho, sigma, tau, xi, ypsilon, have been identified in *E. albertii* (Fiedoruk et al., 2014; Hinenoya et al., 2017b; Lacher et al., 2006; Luo et al., 2011; Ooka et al., 2012, 2015). Although predominantly identified intimin subtype among clinical isolates was sigma followed by beta3 and omicron, the new subtypes have also been gradually reported in *E. albertii* (Hinenoya et al., 2017b; Ooka et al., 2012). In this study, 4 out of the 10 subtypes identified in the 17 *E. albertii* strains were also unknown subtypes (Table1, Fig S1). These data indicate that *E. albertii* express various subtypes of intimin on their surfaces to adhere to host intestine, which does not belong to those predominantly identified in clinically important *E. coli*, such as alpha1 of EPEC O127:H6, and gamma1, epsilon1 and beta1 of EHEC O157, O103 and O26, respectively (Ramachandran et al., 2003). Since intimin might be associated with cell tropisms of host bacteria (Phillips and Frankel, 2000), *E. albertii* might adhere to different intestinal sites from those of clinically important *E. coli*.

Stx2 is the most important virulence factor in EHEC because Stx2-producing EHEC can cause severe diseases such as hemolytic uremic syndrome (HUS) and neurological disorder. Stx2 is encoded on lambdoid phage, whose induction through SOS response is strictly linked for host bacteria to produce Stx2 (Waldor and Friedman, 2005). Clinical isolation of *stx2* gene-positive strain in the case of *E. albertii* has been increasingly reported (Brandal et al., 2005; Etoh et al., 2009; Ooka et al., 2012; Seto et al., 2007). In this study, an *stx2f* gene-positive strain was newly identified. The strain AKT5 produced biologically active Stx2f, whose production could be enhanced by MMC treatment. Furthermore, the production level of Stx2f was higher than that of *E. albertii* strain P2660 which we have previously identified (Hinenoya et al., 2017a) and almost similar to that of EHEC O157:H7 strain Sakai in terms of Vero cell cytotoxicity (Table 2), suggesting that strain AKT5 has higher potential to cause severe diseases than strain P2660. However, unfortunately it cannot be discussed here since detailed clinical information of the patient from which strain AKT5 was isolated is not available.

In summary, this study adds to the evidence base concerning *E. albertii*, mainly through virulence gene characterization. Our studies suggest that *Eccdt-II* gene positive *E. coli* are not true *E. coli*, but rather *E. albertii*. We have demonstrated sequence uniqueness with *E. albertii* and *Eacdt* genes, which may be a successful mechanism for species differentiation, which we are currently working on. All such data are to be welcomed to help with laboratory identification and avoidance of misidentification of other species and *E. albertii*. Our description of virulence characteristics of *E. albertii* further adds to the emerging picture of *E. albertii* as a gastrointestinal pathogen. Further

epidemiological studies are required to understand the clinical importance and properties of *E. albertii*.

Ethical statement

None to declare.

Conflict of interest

None to declare.

Acknowledgement

This work was supported in part by Japanese Society for the Promotion of Science, Japan and Japan Agency for Medical Research and Development, Japan under Grant Numbers 17659131 and JP16jk021007, respectively, to S.Y.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2018.12.003.

References

Abbott, S.L., O'Connor, J., Robin, T., Zimmer, B.L., Janda, M., 2003. Biochemical properties of a newly described *Escherichia* species, *Escherichia albertii*. J. Clin. Microbiol. 41, 4852–4854.

Albert, M.J., Alam, K., Islam, M., Montanaro, J., Rahaman, A.S., Haider, K., Hossain, M.A., Kibriya, A.K., Tzipori, S., 1991. *Hafnia alvei*, a probable cause of diarrhea in humans. Infect. Immun. 59, 1507–1513.

Albert, M.J., Faruque, S.M., Ansaruzzaman, M., Islam, M.M., Haider, K., Alam, K., Kabir, I., Robins-Browne, R., 1992. Sharing of virulence-associated properties at the phenotypic and genetic levels between enteropathogenic *Escherichia coli* and *Hafnia alvei*. J. Med. Microbiol. 37, 310–314.

Beutin, L., Miko, A., Krause, G., Pries, K., Haby, S., Steege, K., Albrecht, N., 2007. Identification of human-pathogenic strains of Shiga toxin-producing *Escherichia coli* from food by a combination of serotyping and molecular typing of Shiga toxin genes. Appl. Environ. Microbiol. 73, 4769–4775.

Brandal, L.T., Tunsjø, H.S., Ranheim, T.E., Løbersli, I., Lange, H., Wester, A.L., 2005. Shiga toxin 2a in Escherichia albertii. J. Clin. Microbiol. 53, 1454–1455.

Brenner, D.J., Davis, B.R., Steigerwalt, A.G., Riddle, C.F., McWhorter, A.C., Allen, S.D., Farmer 3rd, J.J., Saitoh, Y., Fanning, G.R., 1982a. Atypical biogroups of *Escherichia coli* found in clinical specimens and description of *Escherichia hermannii* sp. nov. J. Clin. Microbiol. 15, 703–713.

Brenner, D.J., McWhorter, A.C., Knutson, J.K., Steigerwalt, A.G., 1982b. *Escherichia vulneris*: a new species of *Enterobacteriaceae* associated with human wounds. J. Clin. Microbiol. 15, 1133–1140.

Brenner, D.J., Steigerwalt, A.G., Wathen, H.G., Gross, R.J., Rowe, B., 1982c. Confirmation of aerogenic strains of *Shigella boydii* 13 and further study of *Shigella* serotypes by DNA relatedness. J. Clin. Microbiol. 16, 432–436.

Etoh, Y., Murakami, K., Ichihara, S., Sera, N., Hamasaki, M., Takenaka, S., Horikawa, K., Kawano, K., Takeishi, T., Kuwana, Y., Inoue, A., Nagatsu, Y., Hira, Y., Takahashi, M., Ito, K., 2009. Isolation of Shiga toxin 2f-producing *Escherichia coli* (O115:HNM) from an adult symptomatic patient in Fukuoka prefecture, Japan. Jpn. J. Infect. Dis. 62, 315–317.

Ewing, W.H., 1986. The genus *Escherichia*. In: Edwards, P.R. (Ed.), Edwards and Ewing's Identification of *Enterobacteriaceae*, 4th ed. Elsevier, New York, NY, pp. 93–122.

Farmer 3rd, J.J., Fanning, G.R., Davis, B.R., O'Hara, C.M., Riddle, C., Hickman-Brenner, F.W., Asbury, M.A., Lowery 3rd, V.A., Brenner, D.J., 1985. *Escherichia fergusonii* and *Enterobacter taylorae*, two new species of *Enterobacteriaceae* isolated from clinical specimens. J. Clin. Microbiol. 21, 77–81.

Fiedoruk, K., Daniluk, T., Swiecicka, I., Murawska, E., Sciepuk, M., Leszczynska, K., 2014. First complete genome sequence of *Escherichia albertii* strain KF1, a new potential human enteric pathogen. Genome Announc. 2, e00004–14.

Fox, J.G., Rogers, A.B., Whary, M.T., Ge, Z., Taylor, N.S., Xu, S., Horwitz, B.H., Erdman, S.E., 2004. Gastroenteritis in NF-kappaB-deficient mice is produced with wild-type *Campylobacter jejuni* but not with *C. jejuni* lacking cytolethal distending toxin despite persistent colonization with both strains. Infect. Immun. 72, 1116–1125.

Ge, Z., Feng, Y., Whary, M.T., Nambiar, P.R., Xu, S., Ng, V., Taylor, N.S., Fox, J.G., 2005. Cytolethal distending toxin is essential for *Helicobacter hepaticus* colonization in outbred Swiss Webster mice. Infect. Immun. 73, 3559–3567.

Ge, Z., Rogers, A.B., Feng, Y., Lee, A., Xu, S., Taylor, N.S., Fox, J.G., 2007. Bacterial cytolethal distending toxin promotes the development of dysplasia in a model of microbially induced hepatocarcinogenesis. Cell. Microbiol. 9, 2070–2080.

Hinenoya, A., Naigita, A., Ninomiya, K., Asakura, M., Shima, K., Seto, K., Tsukamoto, T., Ramamurthy, T., Faruque, S.M., Yamasaki, S., 2009. Prevalence and characteristics of cytolethal distending toxin-producing *Escherichia coli* from children with diarrhea in Japan. Microbiol. Immunol. 53, 206–215.

Hinenoya, A., Shima, K., Asakura, M., Nishimura, K., Tsukamoto, T., Ooka, T., Hayashi, T., Ramamurthy, T., Faruque, S.M., Yamasaki, S., 2014. Molecular characterization of cytolethal distending toxin gene-positive *Escherichia coli* from healthy cattle and swine in Nara, Japan. BMC Microbiol. 14, 97.

Hinenoya, A., Yasuda, N., Hibino, T., Shima, A., Nagita, A., Tsukamoto, T., Yamasaki, S., 2017a. Isolation and characterization of an *Escherichia albertii* producing three different toxins isolated from a child with diarrhea. Jpn. J. Infect. Dis. 70, 252–257.

Hinenoya, A., Yasuda, N., Mukaizawa, N., Sheikh, S., Niwa, Y., Awasthi, S.P., Asakura, M., Tsukamoto, T., Nagita, A., Albert, M.J., Yamasaki, S., 2017b. Association of cytolethal distending toxin-II gene-positive *Escherichia coli* with *Escherichia albertii*, an emerging zoonotic pathogen. Int. J. Med. Microbiol. 307, 564–571.

Huys, G., Cnockaert, M., Janda, J.M., Swings, J., 2003. *Escherichia albertii* sp. Nov., a diarrhoeagenic species isolated from stool specimens of Bangladeshi children. Int. J. Syst. Evol. Microbiol. 53, 807–810.

Hyma, K.E., Lacher, D.W., Nelson, A.M., Bumbaugh, A.C., Janda, J.M., Strockbine, N.A., Young, V.B., Whittam, T.S., 2005. Evolutionary genetics of a new pathogenic *Escherichia* species: *Escherichia albertii* and related *Shigella boydii* strains. J. Bacteriol. 187, 619–628.

Lacher, D.W., Steinsland, H., Whittam, T.S., 2006. Allelic subtyping of the intimin locus (*eae*) of pathogenic *Escherichia coli* by fluorescent RFLP. FEMS Microbiol. Lett. 261, 80–87.

Leclerc, H., 1962. Etude biochimique d'enterobacteriaceae pigmentees. Ann. Inst. Pasteur (Paris) 102, 726–741.

Lindsey, R.L., Garcia-Toledo, L., Fasulo, D., Gladney, L.M., Strockbine, N., 2017. Multiplex polymerase chain reaction for identification of *Escherichia coli*, *Escherichia albertii* and *Escherichia fergusonii*. J. Microbiol. Methods 140, 1–4.

Liu, S., Jin, D., Lan, R., Wang, Y., Meng, Q., Dai, H., Lu, S., Hu, S., Xu, J., 2015. *Escherichia marmotae* sp. nov., isolated from faeces of *Marmota himalayana*. Int. J. Syst. Evol. Microbiol. 65, 2130–2134.

Luo, C., Walk, S.T., Gordon, D.M., Feldgarden, M., Tiedje, J.M., Konstantinidis, K.T., 2011. Genome sequencing of environmental *Escherichia coli* expands understanding of the ecology and speciation of the model bacterial species. Proc. Natl. Acad. Sci. U.S.A. 108, 7200–7205.

Maeda, E., Murakami, K., Okamoto, F., Etoh, Y., Sera, N., Ito, K., Fujimoto, S., 2014. Nonspecificity of primers for *Escherichia albertii* detection. Jpn. J. Infect. Dis. 67, 503–505.

McAuley, J.L., Linden, S.K., Png, C.W., King, R.M., Pennington, H.L., Gendler, S.J., Florin, T.H., Hill, G.R., Korolik, V., McGuckin, M.A., 2007. MUC1 cell surface mucin is a critical element of the mucosal barrier to infection. J. Clin. Invest. 117, 2313–2324.

Migula, W., 1895. Bacteriaceae (stabchenbacterien). In: Engler, A., Prantl, K. (Eds.), Die naturlichen pfanzenfamilien, Teil, I., Abteilung Ia. Wilhelm Engelmann, pp. 20–30 Leipzig, Germany.

Murakami, K., Etoh, Y., Tanaka, E., Ichihara, S., Horikawa, K., Kawano, K., Ooka, T., Kawamura, Y., Ito, K., 2014. Shiga toxin 2f-producing *Escherichia albertii* from a symptomatic human. Jpn. J. Infect. Dis. 67, 204–208.

Oaks, J.L., Besser, T.E., Walk, S.T., Gordon, D.M., Beckmen, K.B., Burek, K.A., Haldorson, G.J., Bradway, D.S., Ouellette, L., Rurangirwa, F.R., Davis, M.A., Dobbin, G., Whittam, T.S., 2010. *Escherichia albertii* in wild and domestic birds. Emerg. Infect. Dis. 16, 638–646.

Ooka, T., Seto, K., Kawano, K., Kobayashi, H., Etoh, Y., Ichihara, S., Kaneko, A., Isobe, J., Yamaguchi, K., Horikawa, K., Gomes, T.A., Linden, A., Bardiau, M., Mainil, J.G., Beutin, L., Ogura, Y., Hayashi, T., 2012. Clinical significance of Escherichia albertii. Emerg. Infect. Dis. 18, 488–492.

Ooka, T., Ogura, Y., Katsura, K., Seto, K., Kobayashi, H., Kawano, K., Tokuoka, E., Furukawa, M., Harada, S., Yoshino, S., Seto, J., Ikeda, T., Yamaguchi, K., Murase, K., Gotoh, Y., Imuta, N., Nishi, J., Gomes, T.A., Beutin, L., Hayashi, T., 2015. Defining the genome features of *Escherichia albertii*, an emerging enteropathogen closely related to *Escherichia coli*. Genome Biol. Evol. 7, 3170–3179.

Pandey, M., Khan, A., Das, S.C., Sarkar, B., Kahali, S., Chakraborty, S., Chattopadhyay, S., Yamasaki, S., Takeda, Y., Nair, G.B., Ramamurthy, T., 2003. Association of cytolethal distending toxin locus cdtBwith enteropathogenic Escherichia coliisolated from patients with acute diarrhea in Calcutta, India. J. Clin. Microbiol. 41, 5277–5281.

Phillips, A.D., Frankel, G., 2000. Intimin-mediated tissue specificity in enteropathogenic *Escherichia coli* interaction with human intestinal organ cultures. J. Infect. Dis. 181, 1496–1500.

Priest, F.G., Barker, M., 2010. Gram-negative bacteria associated with brewery yeasts: reclassification of *Obesumbacterium proteus* biogroup 2 as *Shimwellia pseudoproteus* gen. nov., sp. nov., and transfer of *Escherichia blattae* to *Shimwellia blattae* comb. nov. Int. J. Syst. Evol. Microbiol. 60, 828–833.

Ramachandran, V., Brett, K., Hornitzky, M.A., Dowton, M., Bettelheim, K.A., Walker, M.J., Djordjevic, S.P., 2003. Distribution of intimin subtypes among *Escherichia coli* isolates from ruminant and human sources. J. Clin. Microbiol. 41, 5022–5032.

Seto, K., Taguchi, M., Kobayashi, K., Kozaki, S., 2007. Biochemical and molecular characterization of minor serogroups of Shiga toxin-producing *Escherichia coli* isolated from humans in Osaka prefecture. J. Vet. Med. Sci. 69, 1215–1222.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetic analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

Waldor, M.K., Friedman, D.I., 2005. Phage regulatory circuits and virulence gene expression. Curr. Opin. Microbiol. 8, 459–465.

Wilson, K., 2001. Preparation of genomic DNA from bacteria. Curr. Protoc. Mol. Biol Chapter 2:Unit 2.4.

Wirth, T., Falush, D., Lan, R., Colles, F., Mensa, P., Wieler, L.H., Karch, H., Reeves, P.R., Maiden, M.C., Ochman, H., Achtman, M., 2006. Sex and virulence in *Escherichia coli*: an evolutionary perspective. Mol. Microbiol. 60, 1136–1151.

Yamasaki, S., Lin, Z., Shirai, H., Terai, A., Oku, Y., Ito, H., Ohmura, M., Karasawa, T.,

Tsukamoto, T., Kurazono, H., Takeda, Y., 1996. Typing of verotoxins by DNA colony hybridization with poly- and oligonucleotide probes, a bead-enzyme-linked immunosorbent assay, and polymerase chain reaction. Microbiol. Immunol. 40, 345–352.

Yamasaki, S., Asakura, M., Tsukamoto, T., Faruque, S.M., Deb, R., Ramamurthy, T., 2006. Cytolethal distending toxin (CDT): genetic diversity, structure and role in diarrheal disease. Toxin Rev. 25, 61–88.

Young, V.B., Knox, K.A., Pratt, J.S., Cortez, J.S., Mansfield, L.S., Rogers, A.B., Fox, J.G., Schauer, D.B., 2004. In vitro and in vivo characterization of *Helicobacter hepaticus* cytolethal distending toxin mutants. Infect. Immun. 72, 2521–2527.