Supplementary information

Preparation of template DNA for PCR-based screening

The preparation of template DNA for the PCR-based screening of *Escherichia albertii* targeting *lysP* (*E. albertii*-specific sequences in the *lysP* locus[2)]) and *Eacdt* (*E. albertii*-specific *cdt* gene[1)]) was performed using the alkaline-boiling method. The contents adhering to a Seed Swab γ No. 1 (Eiken Chemical Co., Ltd., Tokyo, Japan) were suspended in 10 ml of brain heart infusion (BHI) broth (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), and the suspension was incubated at 37 ℃ for 18 hr. An aliquot (100 μl) was centrifuged at 10,000 × *g* for 10 min, and the supernatant was then removed. The pellet was resuspended in 85 μl of 50 mM NaOH, and the suspension was heated at 100 ℃ for 10 min, neutralized with 15 μl of 1 M Tris-HCl buffer (pH 7.0), and centrifuged again at 10,000 × *g* for 10 min. The collected supernatant was used as template DNA for the screening. The completed templates were stored below 0 ℃ if they were to be used within a short period or below –30 ℃ if they were to be used within several days.

Preparation of template DNA for other PCR procedures

The preparation of template DNA for the PCR identification of *E. albertii*, detection of virulence genes, and *E. albertii* O- and H-genotyping was performed using the alkaline-boiling method. The *E. albertii* isolates/putative isolates were inoculated onto BHI agar (Nissui Pharmaceutical Co., Ltd.), and the media were incubated at 37 ℃ for 16–18 hr. A small number of fresh cells was suspended in 50 μl of 25 mM NaOH, and the suspension was then heated at 95 ℃ for 5 min, neutralized with 4 μl of 1 M Tris-HCl buffer (pH 7.0), and centrifuged at 10,000 × *g* for 5 min. The collected supernatant was used as template DNA for each PCR, and the subsequent handling of samples was as described above.

PCR-based screening targeting *lysP* and *Eacdt*

The PCR-based screening for *lysP* or *Eacdt* was performed using GoTaq Green Master Mix (Promega Corporation, Madison, WI, USA)[1, 2]. The *lysP*/*Eacdt* PCR reaction mixture (10 μl) contained 0.08 μl of 50 μM *lysP* primers or 0.5 μl of 10 μM *Eacdt* primers (Supplementary Table 2; final concentration 0.4 μM [*lysP*] or 0.5 μM [*Eacdt*]), 5 μl of 2× polymerase mixture (final concentration 1×), 0.5 μl of the template DNA prepared above, and PCR-grade water (up to 10 μl). PCR was then conducted using a TaKaRa PCR Thermal Cycler Dice TP650 (Takara Bio Inc., Shiga, Japan) with initial denaturation for 2 min at 95 ℃, followed by 35 cycles of 30 s at 94 ℃, 30 s at 60 ℃ (*lysP*) or 50 ℃ (*Eacdt*), and 30 s at 72 ℃, and ending with 5 min at 72 ℃. After electrophoresis of the amplicons in 2.5% w/v agarose, samples positive for both genes were determined to be *E. albertii*-positive and underwent subsequent isolation.

PCR for *E. albertii* identification

The identification of *E. albertii* was performed using GoTaq Green Master Mix for multiplex PCR targeting *clpX* (sequences common in the *clpX* locus of *E. albertii*, *E. coli*, and *Shigella* as positive controls[2, 3]), *lysP*, and *mdh* (*E. albertii*-specific sequences in the *mdh* locus[2]), supplemented by the PCR assay targeting *Eacdt*[1]. For the triplex PCR, reaction mixture (10 μl) contained 0.08 μl of 50 μM each *clpX* and *lysP* primers (Supplementary Table 2; each final concentration 0.4 μM), 0.06 μl of 50 μM *mdh* primers (Supplementary Table 2; final concentration 0.3 μM), 5 μl of 2× polymerase mixture (final concentration 1×), 0.5 μl of the template DNA prepared above, and PCR-grade water (up to 10 μl). The PCR conditions were the same as those for the *lysP* screening described above. In addition, the PCR assay targeting *Eacdt* was also conducted with the same procedure used for the *Eacdt* screening, except that the PCR cycle was repeated 30 times. After separation of the amplicons as described above, putative isolates judged positive by both PCR assays were identified as *E. albertii*.

Pulsed-field gel electrophoresis (PFGE)

The 38 representative *E. albertii* strains were typed using PFGE to compare their fragment patterns of *Xba*I-digested genomic DNA. Fresh cells were suspended into 1× TE buffer (pH 8.0), and the suspension (McFarland 5) was embedded in 1.0% w/v agarose gel at a capacity ratio of 1:1 to form an agarose plug. After the plug was hardened, in situ lysis of the cells was conducted using 1 mg/ml Proteinase K solution (1 mg of Proteinase K [MACHEREY-NAGEL, GmbH & Co. KG, Düren, Germany] and 10 mg of N-lauroylsarcosine per 1 ml of 0.5 M EDTA buffer [pH 8.0]) at 50 ℃ for 2 hr, and the plug was sliced to the appropriate size for electrophoresis. Subsequent to the deactivation of Proteinase K using 4 mM Pefabloc SC solution (0.96 mg of Pefabloc SC per 1 ml of TE buffer [pH 8.0]), genomic DNA in the agarose plug was digested with 30 U of *Xba*I (Takara Bio Inc.) at 37 ℃ for 4 hr. The digested agarose plugs were sealed in 1.0% w/v PrimeGel Agarose GOLD 3-40K (TaKaRa Bio Inc.; dissolved in the same buffer used for electrophoresis) and electrophoresed in a 0.5× TBE buffer (with thiourea added to 200 μM) using a CHEF DR-III Pulsed Field Electrophoresis System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The analysis conditions were as follows: initial switch time, 2.2 s; final switch time, 54.2 s; voltage, 6 V; included angle, 120°; run time, 19 hr; temperature of cooling module, 14 ℃; and flow rate of pump, 60 l/min. The *Xba*I-digested genomic DNA of *Salmonella* Braenderup H9812 was used as a size marker, and fragment pattern comparisons were based on this marker.

MacConkey agar supplemented with various carbohydrates

To create the lactose, raffinose, salicin-MacConkey (LRS-MAC) agar, 10 g each of lactose (as anhydrate; Nacalai Tesque, Inc., Kyoto, Japan), D-raffinose (as anhydrate; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), and salicin (Tokyo Chemical Industry Co., Ltd.) were added to each liter of

MacConkey basal medium (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). For xylose, rhamnose-MacConkey (XR-MAC) agar, 10 g each of D-xylose (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) and L-rhamnose (as anhydrate; Nacalai Tesque, Inc.) were added to the basal medium per liter. Each medium was prepared according to the manufacturer's instructions and was not sterilized by autoclaving.

Sources of commercially available media used for the examination of biochemical properties

Cellobiose lactose indole β-D-glucuronidase (CLIG) agar was obtained from Kyokuto Pharmaceutical Industrial Co., Ltd. (Tokyo, Japan). Triple sugar iron (TSI) agar, lysine indole motility (LIM) medium, sulfide indole motility (SIM) medium, Voges-Proskauer (VP)-methyl red (VP-MR) broth, and o-nitrophenyl-β-D-galactopyranoside (ONPG) discs were obtained from Nissui Pharmaceutical Co., Ltd. VP medium, Simmon's citrate agar, and Ewing modified malonate broth were obtained from Eiken Chemical Co., Ltd.

References

1) Hinenoya A, Ichimura H, Yasuda N, Harada S, Yamada K, Suzuki M, Iijima Y, Nagita A, Albert MJ, Hatanaka N, Awasthi SP, Yamasaki S. Development of a specific cytolethal distending toxin (*cdt*) gene (*Eacdt*)-based PCR assay for the detection of *Escherichia albertii*. Diagn Microbiol Infect Dis 95, 119–124, 2019. doi: 10.1016/j.diagmicrobio.2019.04.018.

2) Hyma KE, Lacher DW, Nelson AM, Bumbaugh AC, Janda JM, Strockbine NA, Young VB, Whittam TS. Evolutionary genetics of a new pathogenic *Escherichia* species: *Escherichia albertii* and related *Shigella boydii* strains. J Bacteriol 187, 619–628, 2005. doi: 10.1128/JB.187.2.619-628.2005.

3) Oaks JL, Besser TE, Walk ST, Gordon DM, Beckmen KB, Burek KA, Haldorson GJ, Bradway DS, Ouellette L, Rurangirwa FR, Davis MA, Dobbin G, Whittam TS. *Escherichia albertii* in wild and

domestic birds. Emerg Infect Dis 16, 638–646, 2010. doi: 10.3201/eid1604.090695.