

Loss of O157 O Antigenicity of Verotoxin-Producing *Escherichia coli* O157:H7 Surviving under Starvation Conditions

YUKIKO HARA-KUDO,^{1*} MICHIKO MIYAHARA,² AND SUSUMU KUMAGAI¹

Department of Biomedical Food Research, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162-8640,¹ and Division of Microbiology, National Institute of Health Sciences, Setagaya-ku, Tokyo 158-8501, Japan²

Received 27 March 2000/Accepted 29 September 2000

Verotoxin (VT)-producing *Escherichia coli* O157:H7 was culturable on agar media after being left in water for 21 months. However, there were a number of colonies which had lost O157 O antigenicity. These colonies produced VTs, which are pathogenic to humans. These observations suggest that the immunologic methods based on O157 O antigenicity are unable to detect and isolate VT-producing *E. coli* in foods and other environments if the organism has been under starvation conditions for a long period.

Transmission of *Escherichia coli* O157:H7 via water, such as drinking water and lake water, is important in *E. coli* O157:H7 infection (1, 4, 7, 9, 11, 14). It is suggested that water is contaminated with *E. coli* O157:H7 by humans and animals harboring the organism. There are several studies showing survival of *E. coli* O157:H7 in water for a long time under starvation conditions (12, 15, 16). For *E. coli* serotypes other than *E. coli* O157, expression of genes has been shown to be changed relative to the survival of the organism in water (3, 10). However, for *E. coli* O157, it is uncertain whether such changes occur with survival.

In the present study, we investigated the viability and change in the biochemical and antigenic properties characteristic of *E. coli* O157:H7 in distilled water (DW) and phosphate-buffered saline (PBS), which were chosen as a model for starvation conditions.

The bacterial strains (given by K. Tamura, Department of Bacteriology, National Institute of Infectious Diseases) used in this study are shown in Table 1. The 19 strains were cultured separately in 10 ml of tryptic soy broth (TSB; Difco, Detroit, Mich.) for 18 h at 37°C. The TSB cultures were centrifuged at 4,000 × *g* for 20 min. The supernatant was removed, and the pellet was washed three times with 10 ml of PBS (Nissui Co. Ltd., Tokyo, Japan) or DW (Nihon Millipore Ltd., Tokyo, Japan). Finally, the cells of all 19 strains, washed with PBS, were resuspended in 10 ml of PBS at 10⁷ CFU/ml. For three strains (A, B, and C), cells washed with DW were resuspended in 10 ml of DW at 10², 10³, and 10⁵ CFU/ml. They were then incubated statically at 4 or 18°C.

E. coli O157:H7 bacteria in PBS and in DW were incubated for 230 and 635 days, respectively. During incubation, 0.1 ml of the cell suspension was taken and serially diluted with PBS, and 0.5 ml of the suspension was plated onto five plates of tryptic soy agar (Difco). The colonies formed on the plates were counted after 18 h of incubation at 37°C to determine cell numbers.

Cell numbers decreased gradually with time in PBS at 4°C (Table 2). The cell numbers for P and the other 14 strains (B, C, D, F, G, J, K, L, M, N, O, Q, R, and S) decreased to below

the detectable level (2 CFU/ml) on days 105 and 230, respectively. The remaining three strains (A, E, and H) survived till day 230. The numbers for some of these strains tended to decrease with time at 18°C, but the decrease was not as remarkable as that at 4°C.

The numbers of bacteria decreased with time in DW also at 4°C (Fig. 1a). They tended to decrease more slowly when 10⁵ CFU/ml was inoculated than when 10² and 10³ CFU/ml were inoculated. When incubated at 18°C (Fig. 1b), *E. coli* O157:H7 that had been inoculated at the levels of 10² and 10³ CFU/ml decreased to fewer than 2 CFU/ml within 48 days, but those bacteria inoculated at the level of 10⁵ CFU/ml decreased by less than 1 order of magnitude during 48 days of incubation (Fig. 1b). The fate of the *E. coli* O157:H7 population depended on the initial number, indicating the importance of bacterial concentration in water. This phenomenon may relate to the interaction of bacterial cells. As found on PBS, *E. coli* O157:H7 at high cell concentrations survived better at 18°C than at 4°C (Fig. 1a and b).

Colonies on tryptic soy agar at day 230 of incubation in PBS and those at days 277 and 635 of incubation in DW were tested for O antigenicity with an agglutination kit (UNI; Oxoid) and

TABLE 1. Strains of *E. coli* O157:H7 used in this study

| Strain | Reference no. | Source | VT production |
|--------|---------------|----------------|---------------|
| A | 2 | Patient | VT1, VT2 |
| B | 126 | Beef | VT2 |
| C | 23 | Patient | VT1, VT2 |
| D | 212 | Patient | VT1, VT2 |
| E | 298 | Patient | VT2 |
| F | 133 | Beef | VT1, VT2 |
| G | 1291 | Salad | VT1, VT2 |
| H | KE5 | Patient | VT1, VT2 |
| I | 758 | Patient | VT1, VT2 |
| J | 1102 | Beef | VT1, VT2 |
| K | 1646 | Salad | VT2 |
| L | 970056 | Radish sprouts | VT1, VT2 |
| M | 18 | Patient | VT1, VT2 |
| N | 42 | Patient | VT1, VT2 |
| O | 60 | Patient | VT1, VT2 |
| P | 132 | Beef | VT2 |
| Q | 174 | Patient | VT2 |
| R | 307 | Patient | VT1, VT2 |
| S | 434 | Patient | VT1, VT2 |

* Corresponding author. Mailing address: Department of Biomedical Food Research, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Phone: 81 3 5285 1111. Fax: 81 3 5285 1176. E-mail: ykudo@nih.go.jp.

TABLE 2. Fate of *E. coli* O157:H7 strains in PBS

| Temp (°C) | Day | No. of cells (log CFU/ml) for each strain | | | | | | | | | | | | | | | | | | |
|-----------|-----|-------------------------------------------|-----------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| | | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S |
| 4 | 0 | 6.4 | 6.1 | 6.5 | 6.5 | 6.5 | 6.5 | 6.7 | 7.0 | 6.5 | 6.4 | 6.3 | 6.5 | 5.9 | 6.1 | 6.4 | 6.4 | 6.7 | 6.6 | 6.6 |
| | 2 | 5.8 | 5.2 | 5.8 | 5.8 | 5.8 | 5.3 | 5.5 | 6.1 | 5.9 | 5.8 | 5.8 | 6.2 | 5.6 | 5.7 | 6.0 | 5.9 | 6.0 | 5.6 | 5.8 |
| | 21 | 5.1 | 4.9 | 5.2 | 5.2 | 5.0 | 5.2 | 5.3 | 6.0 | 4.4 | 5.1 | 5.0 | 5.4 | 5.0 | 4.1 | 5.4 | 4.8 | 5.4 | 5.4 | 4.9 |
| | 42 | 5.4 | 5.2 | 4.9 | 5.3 | 5.3 | 5.5 | 5.5 | 5.8 | 5.4 | 5.1 | 5.1 | 5.8 | 4.7 | 5.2 | 5.5 | 4.5 | 5.7 | 5.3 | 4.3 |
| | 105 | 3.8 | 2.9 | 2.2 | 4.5 | 3.7 | 3.9 | 2.2 | 5.1 | 1.3 | 1.5 | 1.2 | 4.0 | 2.0 | 2.0 | 1.0 | ND | 4.0 | 3.5 | 1.9 |
| | 230 | 1.0 | ND ^a | ND | ND | 1.7 | ND | ND | 4.7 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 18 | 0 | 5.8 | 5.4 | 5.9 | 6.2 | 6.4 | 6.0 | 6.1 | 6.8 | 6.4 | 6.3 | 6.0 | 6.8 | 5.7 | 5.9 | 6.5 | 5.7 | 6.2 | 5.7 | 6.2 |
| | 2 | 5.8 | 5.0 | 6.1 | 5.4 | 6.0 | 5.5 | 6.1 | 6.2 | 6.0 | 5.9 | 5.5 | 7.0 | 6.1 | 5.1 | 6.1 | 6.1 | 6.0 | 5.7 | 5.9 |
| | 21 | 5.0 | 3.6 | 5.4 | 5.3 | 5.2 | 5.8 | 5.9 | 6.1 | 6.4 | 5.3 | 4.0 | 5.3 | 4.3 | 4.3 | 5.7 | 3.6 | 5.1 | 5.3 | 5.4 |
| | 42 | 5.4 | 4.9 | 5.3 | 5.4 | 5.5 | 5.4 | 5.4 | 5.7 | 5.4 | 5.4 | 4.1 | 4.3 | 4.3 | 4.9 | 5.4 | 4.0 | 4.9 | 4.6 | 5.3 |
| | 105 | 5.0 | 4.9 | 4.8 | 4.6 | 4.9 | 4.9 | 4.8 | 4.4 | 5.0 | 5.3 | 4.3 | 4.6 | 4.7 | 4.6 | 4.6 | 4.7 | 4.9 | 4.6 | 4.9 |
| | 230 | 5.6 | 4.6 | 5.0 | 4.8 | 4.7 | 5.3 | 5.4 | 5.0 | 5.0 | 5.6 | 4.6 | 4.6 | 5.0 | 5.3 | 4.3 | 4.5 | 4.9 | 4.9 | 5.4 |

^a ND, not detected (<0.3 log CFU/ml).

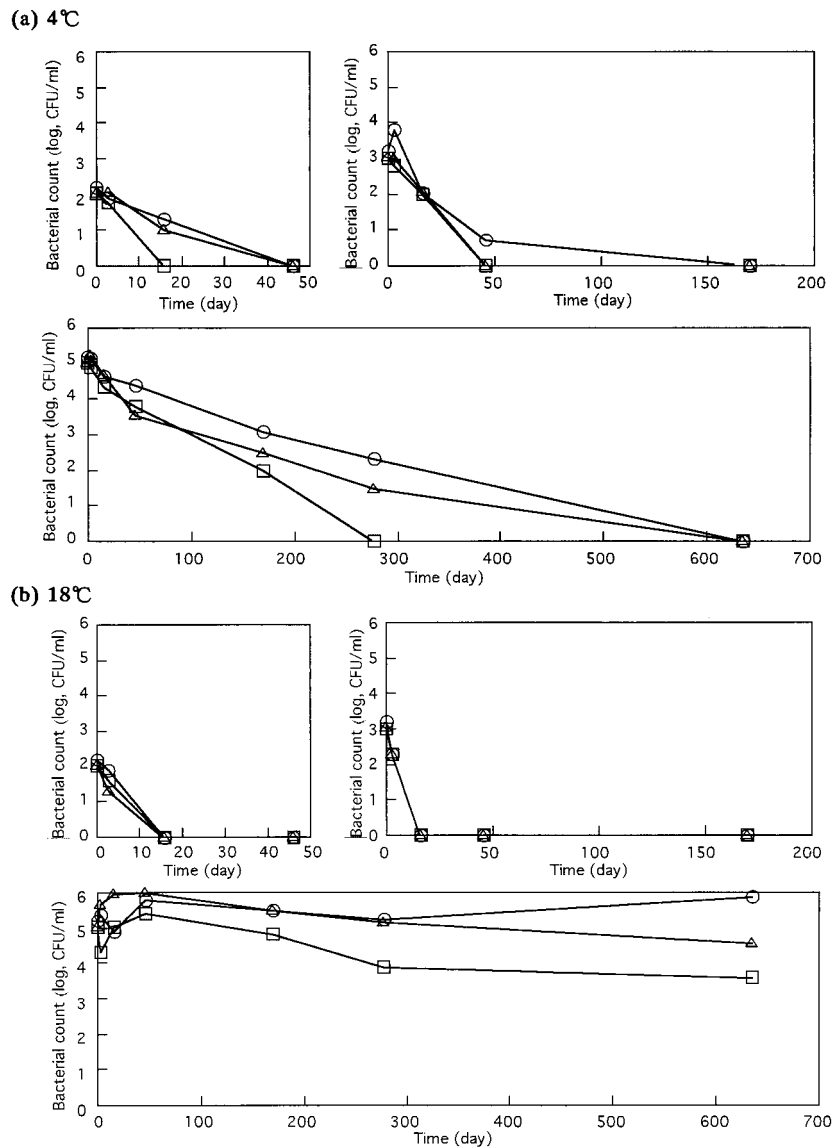


FIG. 1. Fate of three *E. coli* O157:H7 strains in DW at 4°C (a) and 18°C (b). Strain and inoculation level symbols: ○, strain A; □, strain B; △, strain C.

O157 antiserum (Denka Seiken Co. Ltd., Tokyo, Japan) without heat treatment of the colonies. The colonies were also tested for cellobiose and lactose fermentation and indole- and glucuronidase-positive reactions with cellobiose-lactose-indole-D-glucuronidase agar (Kyokuto, Tokyo, Japan) culture. Nonagglutinated colonies were found in strain A at 4°C and strains B, K, P, Q, R, and S at 18°C on day 230 in PBS and in strain A at 18°C on days 277 and 635 in DW. Shimizu et al. (13) showed that *orf2* is an important gene for the synthesis of the O157 O antigen. To confirm genetically the loss of O157 antigenicity by detection of *orf2*, the colonies were inoculated in TSB and cultured at 37°C for 18 h. The TSB culture was then centrifuged at 40,000 × *g* for 20 min. The supernatants were assayed for the presence of verotoxins (VTs) using a latex agglutination assay kit (Denka Seiken) by following the manufacturer's instructions. Cell pellets were assayed for *orf2* genes and VT genes by PCR according to the method of Shimizu et al. (13) and the manufacturer's instructions (O157 PCR screening set; Takara, Tokyo, Japan), respectively. The amplified gene was analyzed by electrophoresis in a 2% agarose gel. Table 3 shows the changes in properties characteristic of inoculated strains after incubation in PBS at 4 or 18°C for 230 days or in DW at 18°C for 277 and 635 days. A number of isolates of strains A, B, K, Q, and S lost O157 O antigenicity, although the *orf2* gene encoding O157 O antigen existed in these isolates. However, some isolates of strains P and R lost the *orf2* gene as well as O157 O antigenicity (data not shown). These findings suggest that *E. coli* O157 may occasionally lose O157 O antigenicity not only with the loss of the *orf2* gene but also without it.

The lost O157 O antigenicity was not recovered by culture in TSB. These strains were confirmed to possess such properties of *E. coli* O157 as cellobiose nonfermentation, lactose fermentation, and indole- and β-glucuronidase-positive reactions. The VT gene was maintained in these strains, which possessed the gene initially. The strains maintained VT production except for the VT2 production of strain A. It was suggested that the expression of the VT2 gene was inhibited in strain A.

Many O antigen serotypes of *E. coli* are known to produce VTs or to be pathogenic. The *E. coli* O157:H7 strain which lost O157 O antigenicity was tested for agglutination to the other O antigen antisera (Denka Seiken). However, the O antigen of the *E. coli* O157:H7 strain which lost O157 O antigenicity did not change to the other types, such as O1, O6, O8, O15, O18, O20, O25, O26, O27, O28, O29, O44, O55, O63, O78, O86, O111, O112, O114, O115, O119, O124, O125, O126, O127, O128, O136, O142, O143, O144, O146, O148, O151, O152, O153, O157, O158, O159, O164, O166, O167, O168, and O169 of pathogenic *E. coli*.

Wang and Doyle (15) showed changes in outer membrane proteins of *E. coli* O157:H7 cells surviving in water. In the present study, we found a loss of immunologic response against O157 O antigen. O antigenicity is a very important characteristic for detection of O157, a most frequently isolated type of enterohemorrhagic *E. coli* (EHEC) (6). Many kinds of detection kits and systems utilizing O157 O antigenicity have been developed (2, 5, 8) and widely used to detect a small number of *E. coli* O157 bacteria in food and environmental samples contaminated with a large number of competitive bacteria. However, a method which does not depend on immunologic techniques should be used to test for EHEC in foods and other environments, when the organism is suspected to have been under starvation conditions for a long period.

TABLE 3. Change in properties characteristic of *E. coli* O157:H7 after incubation in water^a

| Strain | Results before incubation | | | | | | | | | | Results after incubation | | | | | | | | | | |
|--------|---------------------------|----------------------|---------|---------|-----------|-----------------------|---------------------------|--------------------------|--------------------------|---------------------------|--------------------------|-------|---------|---------|-----------|-----------|---------|---------|-----------|-----------|-------|
| | O157 O antigenicity | | | | | Incubating conditions | | | | | O157 O antigenicity | | | | | Orf2 | | | | | |
| | Antiserum | Latex | PCR-VT1 | PCR-VT2 | Latex-VT1 | Latex-VT2 | In PBS at 4°C (230 days) | In DW at 18°C (277 days) | In DW at 18°C (635 days) | In PBS at 18°C (230 days) | Antiserum | Latex | PCR-VT1 | PCR-VT2 | Latex-VT1 | Latex-VT2 | PCR-VT1 | PCR-VT2 | Latex-VT1 | Latex-VT2 | |
| A | + | (10/10) ^b | + | (10/10) | + | + | In PBS at 4°C (230 days) | + | (4/8) | + | (4/8) | + | (4/4) | + | (4/4) | + | (4/4) | + | (4/4) | + | (4/4) |
| B | + | (10/10) | + | (10/10) | + | + | In DW at 18°C (277 days) | + | (4/8) | + | (4/8) | + | (0/4) | + | (0/4) | + | (0/4) | + | (0/4) | + | (0/4) |
| K | + | (10/10) | + | (10/10) | + | + | In DW at 18°C (635 days) | + | (0/3) | + | (0/3) | + | (3/3) | + | (3/3) | + | (3/3) | + | (3/3) | + | (3/3) |
| Q | + | (10/10) | + | (10/10) | + | + | In PBS at 18°C (230 days) | + | (3/3) | + | (3/3) | + | (0/3) | + | (0/3) | + | (0/3) | + | (0/3) | + | (0/3) |
| S | + | (10/10) | + | (10/10) | + | + | In PBS at 18°C (230 days) | + | (0/3) | + | (0/3) | + | (3/3) | + | (3/3) | + | (3/3) | + | (3/3) | + | (3/3) |

^a O157 O antigenicity, agglutination test for anti-O157 serum and a latex agglutination kit; Orf2, detection of the *orf2* O157 lipopolysaccharide gene by PCR; PCR-VT1, detection of the VT1 gene by PCR; Latex-VT1, detection of VT1 using the reversed passive latex agglutination test; +, positive reaction; -, negative reaction.

^b Number of colonies with reactions/number of colonies tested.

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