

# Minimum and Maximum Temperatures for Growth and Verotoxin Production by Hemorrhagic Strains of *Escherichia coli*

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## ABSTRACT

The influence of temperature on growth and verotoxin production by *Escherichia coli* strains was studied in brain heart infusion (BHI) broth both in shake cultures at various temperatures and in a temperature-gradient incubator. All strains of *E. coli* surveyed grew from at least 10 to 45°C, with some strains growing at 8°C. Verotoxin production (determined using the Vero cell-assay system) was a function of both temperature and time, with the highest titers produced at temperatures supporting the fastest growth (based on days to visible turbidity) and highest viable cell counts. However, for strains producing verotoxin, toxin production was detected at any temperature supporting growth. Three strains (of 16 tested) increased 1000-fold in viable count in 4 to 6 days at 10°C. The data presented here indicate that most *E. coli* strains surveyed can easily grow at ca. 10°C and thus suggest the potential for growth in temperature-abused refrigerated foods.

Key words: Verotoxigenic *E. coli*, isolation, psychrotrophic growth

Over the last 10 years, hemorrhagic *E. coli*, in particular the O157:H7 serotype, has become recognized as a significant human pathogen associated with foods (5,8,18,21,23). The disease syndrome includes hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura (19,22,24). While the pathogen has been associated mainly with undercooked hamburger and beef as vehicles of transmission (1,2,13), outbreaks also have been associated with raw milk (13,14), unpasteurized apple cider (3), yogurt (16) and an unchlorinated municipal water supply (25). Hemorrhagic *E. coli* serotype O157:H7 appears to be a new strain which first gained the ability to produce attaching and effacing adherence and then acquired phage-encoded genes for Shiga-like toxin production (27). Hemorrhagic *E. coli* (properly enterohemorrhagic *E. coli*, designated EHEC) is one of at least four groupings of pathogenic *E. coli* (17). The virulence mechanisms and toxins of each group vary. For EHEC, the virulence mechanism is a combination of attaching and effacing

adherence to the large bowel (10) and the production of Shiga-like toxins (also known as verotoxins [VT]). EHEC strains can produce either VT1 (found predominantly in cell lysates) and/or VT2 (found in culture filtrates) (17). VT1 and VT2 are detected by the cytotoxicity to HeLa or Vero cells and act by inactivating protein synthesis (17).

Control of pathogens in foods has traditionally depended on low temperatures ( $\leq 5^\circ\text{C}$ ). However, food-borne pathogens including *Yersinia enterocolitica*, *Listeria monocytogenes*, *Aeromonas hydrophila*, and nonproteolytic *Clostridium botulinum* can grow at low temperatures (20). Other food-borne pathogens previously classified as mesophilic are showing psychrotrophic growth: e.g., *Salmonella* (4) and *Bacillus cereus* (15). Olsvik and Kapperud reported that an *E. coli* strain could grow and form heat-stable toxin in broth and broth with cream at 4°C (18). It has long been observed that the genus *Escherichia* is often isolated from refrigerated dairy and other food products (28).

The upper temperature of growth of mesophilic and psychrotrophic food-borne bacteria is important, since food is often held warm at temperatures near the maximum temperature of growth of these bacteria. In addition, an elevated incubation temperature (45°C) is recommended for the enrichment of various samples for the isolation of *E. coli* and fecal coliforms (9,26). Doyle and Schoeni reported that the strain of *E. coli* O157:H7 they tested (strain 933) grew poorly in the temperature range of 44 to 45.5°C (6), while Raghubeer and Matches observed that the single strain of *E. coli* O157:H7 (human isolate) they tested would not grow above 41.0°C within 48 h (21). Thus, any procedure which utilized an elevated-temperature enrichment step would most likely preclude isolation of these bacteria.

Knowledge of the minimum and maximum temperatures of growth for *E. coli* is important in food preservation and for detection of this bacterium. In addition, many of the studies report findings based on single strains. It was the purpose of this study (a) to determine the minimum and maximum growth temperatures for a series of *E. coli* strains including the O157:H7 serotype, and (b) to determine the influence of temperature on verotoxin production.

<sup>†</sup> Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

## MATERIALS AND METHODS

### Cultures

The sources of bacterial strains along with their serotypes and original designations are as follows: (A) *E. coli* Reference Lab., Penn State University, University Park, PA: O157:H7, 88-1559; O157:H7, 88-0225; O157:H7, 88-1558; O157:H7, 90-0105; O157:H7, 88-1457; O157:H7, 90-1772; O157:H7, 90-2152; O157:H7, #1; O157:H7, #3, O111:NM, 88-0015; O145:NM, 88-0963; O145:NM, 87-1009; O113:H21, 88-0632; O91:H21, 83-0625; O22:H8, 90-0327; O4:NM, 88-0545; O103:H2, 87-1368; and O5:NM, 85-0587. (B) Centers for Disease Control, Atlanta, GA: O157:H7, 3417-85; O157:H7, 86-24; O157:H7, C984; O157:H7, EDL 931; O157:H7, A9124-1; O157:H7, A8959-C7; O157:H7, A9218-C1; O157:H7, C9490 (ENT) (1993 outbreak isolate); O26:H11, 1801-72; O26:H11, 3359-70; and O26:H11, 2239-69. (C) Food Safety and Inspection Service, Beltsville, MD: O157:H7, 933; O157:H7, 45753-35; O157:H7, 45753-32; and O157:H7, 45750. (D) M. Doyle, University of Georgia, Athens, GA: O157:H7, 932; O157:H7, 32381; and O157:H7, 505B. (E) D. Thayer, USDA, Philadelphia, PA: O157:H7, 93446; O157:H7, 93-569, and O157:H7, ATCC 43889. (The serotypes above have been associated with hemorrhagic colitis and produced verotoxin [10]; however, except where indicated, we did not screen the specific isolates for their ability to produce verotoxin.) (F) Uniformed Services, University of Health Services, Bethesda, MD: serotype unknown, K-12 (C600). (G) M. Haas, USDA, Philadelphia, PA: serotype unknown, J53. Media were inoculated to yield a starting count of ca.  $\log_{10}$  3 CFU/ml from an appropriate dilution (made in 0.1% peptone water) of an overnight culture (ca. 18 h at 37°C) of the strain grown in brain heart infusion broth (BHI, Difco Laboratories, Inc., Detroit, MI).

### Maximum and minimum growth temperatures

To determine the minimum and maximum temperatures of growth and verotoxin production, special temperature-gradient tubes (L-shaped tubes) containing BHI were inoculated to yield a starting viable cell count of ca.  $\log_{10}$  3 CFU/ml and incubated with shaking (setting 4; 40 oscillations/min) in a temperature-gradient incubator (model TN-3F, Advantec, Dublin, CA) set from 5 to 50°C. At daily intervals, the tubes were observed for turbidity; when visible turbidity was observed, aliquots were removed for viable cell counts and verotoxin quantitation. Viable cell counts were determined by surface plating (model D Spiral Plater, Spiral Biotech, Bethesda, MD) onto duplicate tryptic soy agar (TSA, Difco) plates; serial dilutions were made as needed in 0.1% peptone (Difco) water. Plates were counted by a Laser colony-counting system (Spiral Biotech) after 18 to 24 h incubation at 28°C. At the lower temperatures, a second aliquot was removed for analysis 1 to 3 days after the first sampling.

### Minimum temperature

The minimum temperature of growth was also determined by incubating some of the strains in flasks of BHI broth (100 ml per 250-ml flask; 150 rpm) in Psychrotherm incubators (model G26, New Brunswick Scientific, Edison, NJ) set at 15, 12, 10, 8, and 5°C. At intervals, aliquots were removed for determination of viable cell counts by plating on TSA as described above. Growth responses were determined at the individual temperatures and where growth occurred (at least a 3-log unit increase in viable count observed), lag time and exponential growth rates were calculated by Abacus (an iterative, non-linear, regression program, W. Damert, USDA, Philadelphia, personal communication) and the Gompertz equation (7).

TABLE 1. Growth kinetics and lowest temperature of growth for *E. coli* strains.

Strain	Gradient Incubator		Shake Cultures		
	Growth Observed at °C	No Growth at °C	Lowest Temperature (°C) at Which Growth Occurred	Lag Time in Days at This Temperature	T <sub>1000</sub> ***, in Days at This Temperature
88-1559	9.2	7.3	10*	4.0	7.7
88-0225	9.4	7.4	10*	1.5	4.0
88-1558	9.2	7.3	8**	3.9	9.2
A9124-1	9.4	7.4	10*	5.8	15.7
933	11.6	9.2	10*	8.0	10.9
A9218-C7	11.3	9.4	10*	14.4	23.7
C9490(ENT)	9.4	7.4	10*	3.3	9.7
#1	9.2	7.3	10*	3.1	6.6
#3	9.4	7.4	10*	1.1	5.7
88-1457	9.4	7.4	10*	7.4	12.3
1801-72	11.6	9.2	10*	8.7	13.5
90-0105	9.2	7.3	10*	3.8	7.5
3359-70	7.3	not done	10*	6.8	12.2
90-1772	9.2	7.3	8**	5.0	12.9
90-2152	9.2	7.4	8**	33.3	46.4
K12	9.2	7.4	8**	15.6	26.1

\*Growth at 10 but not at 8°C; \*\*growth at 8 but not at 5°C; \*\*\*time for a 1000-fold increase in viable count.

### Maximum temperature

The ability of the strains to grow at 42 and 45°C in a circulating water bath was evaluated by incubating duplicate tubes (without shaking) of BHI and EC broth (Difco) with Durham tubes at those temperatures. At daily intervals (up to 3 days), the tubes were observed for turbidity (BHI and EC broth cultures) and gas production (EC broth cultures only).

### Growth temperature and verotoxin production

Shake flasks of BHI (100 ml per 250-ml flask) were inoculated with strain A9124-1 to yield a starting count of  $\log_{10}$  3 CFU/ml and incubated with shaking (150 rpm) at 37, 12, and 10°C. At appropriate intervals during incubation, aliquots were removed for viable cell determination and verotoxin titer. The experiment was done twice, though sampling times were not identical.

### Vero-cell assay

Samples for assay were prepared by removing 2 ml from the BHI cultures grown either in the Gradient Incubator or the shake flasks and centrifuging for 10 min at setting 5 in an IEC Clinical Centrifuge (Intl. Equipment Co., Needham Hts., Mass) at room temperature. The supernatant was then passed through an 0.2  $\mu$ m syringe filter (low protein binding, Cat. no. 190-2520, Nalgene Co., Rochester, NY). The cell-free supernatants were used for verotoxin assay either immediately or frozen at minus 20°C until the titer could be determined.

Activity against Vero cells was assayed using the technique of Konowalchuk et al. (12) and Speirs et al. (23). Two plates per strain of bacteria were used for most assays (where a very high titer was anticipated, usually for cultures grown at higher temperatures, a third plate was prepared). The concentration of suspended Vero cells was  $10^5$  to  $10^6$ /ml; 100  $\mu$ l of suspended Vero cells were placed into 96-well microtiter plates and incubated for 18 to 24 h at 37°C, 96% relative humidity, and 5% CO<sub>2</sub>. Samples of the cell-free supernatant (100  $\mu$ l) were placed in the first well of the plate (1:2 dilution) and were diluted two-fold across the plate(s). The plates were then incubated as above. After 72 h incubation, the wells were examined for the presence of living and dead cells. A positive toxin response was defined as 50% of the Vero cells being dead and detached. The titer of verotoxin in a sample was expressed as the reciprocal of the end-point dilution.

## RESULTS AND DISCUSSION

### Temperature gradient studies

Initial experiments with strains of *E. coli* (Table 1) in the temperature-gradient incubator set over a range of 5 to 50°C indicated that most of the strains grew between ca. 10°C (14 of 16 strains tested) and 45°C (growth at the high temperatures to be shown below). Because of the importance of the low temperatures in the refrigerated holding of foods and the use of high temperature(s) in procedures for detection and enumeration of this bacterium from foods as well as the warm holding of foods, we studied the growth response of these strains in greater detail at low and high temperatures.

### Low temperature studies

The ability to grow at low temperatures was studied in greater detail by incubating shake cultures at 5, 8, 10, 12, and 15°C. This allowed observation of growth/no growth temperatures as well as calculation of lag times and  $T_{1000}$  at the lowest temperature at which growth occurred (Table 1). The minimum growth temperatures observed for cultures incubated in the temperature-gradient incubator agreed with those observed in shake cultures. At temperatures below the minimum (Table 1), the number of viable cells decreased to < 21 CFU/ml (the lower limit of detection). The  $T_{1000}$  (time for a 1000-fold increase in viable count) represents the influence of temperature on both lag and generation times.

### High temperature responses

The ability of all strains to grow at the elevated temperatures of 42 and 45°C was studied in both BHI and EC broths. All strains grew at both temperatures in BHI broth (visible turbidity); all but six grew and produced gas in EC broth at 42 and 45°C (Table 2). Growth at these elevated temperatures was a function of the medium used, with BHI being more permissive than EC broth. However, the elevated-temperature isolation procedure for *E. coli* generally employs 45°C with the selective EC broth (9,26). Under these conditions, three strains (A9218-C1, A9124-1, and 2239-69) would have gone undetected, one strain (932) would have required a second day of incubation, and two strains (3417-85 and A8959-C7) gave a positive and a negative reaction in the pair of tubes. Of the six strains giving an atypical response in EC broth, five were O157 serotype (of 23 of this serotype screened). Thus, one day of incubation at 45°C in EC broth would be a useful isolation and detection technique for most (18 of 23 or 78%) O157 serotype strains. The data in Table 2 indicate that

TABLE 2. Different responses of *E. coli* strains tested in EC broth at 42 and 45°C.

Strain	Response
A9218-C1	At 45°C, no growth in 3 days; viable count < 21 CFU/ml. At 42°C, one tube gas + in 3 days; viable count in second tube < 21 CFU/ml.
A9124-1	At 45°C, no growth in 3 days; viable count < 21 CFU/ml. At 42°C, one tube gas + in 3 days; viable count in second tube < 21 CFU/ml.
3417-85	At 45°C, growth in both tubes, gas in one tube.
932	At 45°C, growth in one day, gas produced in 2 days.
A8959-C7	At 45°C, gas produced in one tube; viable count < 21 CFU/ml in second.
2239-69	At 45°C, no gas produced in 3 days; viable count remained at inoculum level of $\log$ 3 CFU/ml. At 42°C, one tube gas + in one day; viable count of second tube was $\log$ 1.3 CFU/ml at day 3.

45°C is not useful for recovering all of the O157 serotype when EC broth is used. In our study, the single strain (932) employed by Doyle and Schoeni (6), which they reported grew poorly at 44 and 45.5°C, grew in both BHI and EC broths in one day but required a second day to be gas positive in EC broth. Raghubeer and Matches also indicated that temperatures above 41°C would not support the growth of the O157:H7 serotype, but their study involved only a single strain (21).

#### Verotoxin production

The influence of temperature on production of verotoxin by an individual *E. coli* strain was studied in cultures grown both in shake flasks and in a temperature-gradient incubator. Strain A9124-1 was grown in BHI shake cultures at 37, 12, and 10°C; at intervals during incubation, aliquots were removed for viable cell count and verotoxin titer determination. Data from individual experiments are presented in Fig. 1. Verotoxin was produced at all three temperatures, with high titers being produced even at the lower temperatures after extended periods of incubation. The amount of toxin was related to the cell yield, with highest cell count and highest titer observed at 37°C.

A temperature-gradient incubator was used to screen the 16 strains of *E. coli* cultures (Table 1) for minimum and maximum temperatures for growth and verotoxin production. Data for three strains are given in Table 3. Three strains (two O111:NM strains 90-2152 and 90-1772) and K12 did not produce verotoxin at any temperature. Several points are noted from the data in Table 3. Verotoxin is formed at all temperatures, with the highest titers at the highest growth temperatures. For a given strain, comparable cell counts at different temperatures did not yield comparable titers: higher titers are formed at the higher temperatures. Verotoxin production appears to be a function of both growth temperature and time (see Fig. 1). At

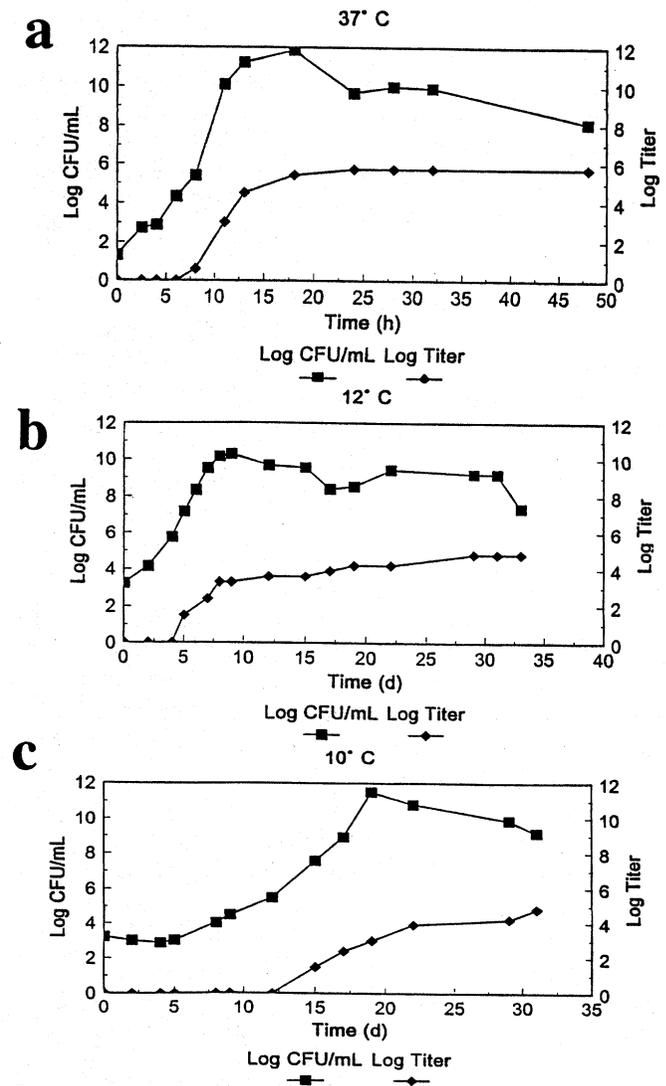


Figure 1. Growth and verotoxin production by *E. coli* A9124-1 at various temperatures. a, 37°; b, 12°; c, 10°.

TABLE 3. Growth and verotoxin production at various temperatures by three strains of *E. coli* in BHI broth incubated in the temperature gradient incubator.

Strain #1 (O157:NM)				Strain A9128-C7 (O157:H7)				Strain 88-1457 (O26:H11)			
Temp. °C	Days*	Log Count	Verotoxin Titer	Temp. °C	Days*	Log Count	Verotoxin Titer	Temp. °C	Days*	Log Count	Verotoxin Titer
49.4	3	6.39	2,048	43.0	1	9.02	2,097,152	45.0	1	9.51	8,192
46.9	1	8.21	>16,777,216	37.2	1	10.14	2,097,152	43.0	1	10.12	16,384
44.9	2	9.10	1,048,576	32.0	1	10.15	2,097,152	38.6	1	10.44	8,192
33.8	1	10.06	65,536	28.4	1	10.78	2,097,152	34.6	1	10.13	4,096
30.4	1	9.87	16,384	24.4	1	10.96	262,144	29.6	1	10.02	4,096
24.4	1	10.35	1,024	22.4	1	10.49	2,048	24.4	1	10.20	512
22.0	1	9.05	64	21.2	1	9.8	64	22.4	1	10.02	256
19.4	1	7.54	8	19.6	2	11.65	8,192	21.2	1	9.04	32
	2	10.59	2,048	18.2	2	10.75	512	18.2	1	9.76	64
17.9	2	10.04	1,024	17.0	2	9.88	16	17.0	2	8.77	32
16.6	2	9.13	128		3	11.46	4,096	15.5	3	8.67	8
15.0	2	7.17	8	15.5	3	10.42	32	13.4	5	9.26	8
	3	9.90	1,024	13.4	6	10.57	2,048				
13.4	4	8.56	256	11.3	8	9.15	16				
11.6	6	8.24	128								
9.2	15	9.0	1,024								

\*Days to visible turbidity and/or sampling.

present, there is no evidence that preformed verotoxin in foods causes disease; however, work by Kittell et al. has indicated that purified toxin was stable to heat (survived 60 min at 75°C and 5 min at 80°C) (11). Thus, any toxin formed in food during holding at temperatures from 10 to 49°C could retain activity during most cooking operations.

In conclusion, the results presented here indicate that some *E. coli* can grow and produce verotoxin in BHI at temperatures as low as 10°C (see Table 3 and Fig. 1) and as high as 49°C (Table 3). Foods are often held in this temperature range, despite recommended good handling practices. Extrapolation of our findings to foods suggests that any food containing pathogenic *E. coli*, including the hemorrhagic strains, held within this temperature range could generate an increased health hazard to consumers.

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