APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1995, p. 2037–2039 Vol. 61, No. 5
0099-2240/95/$04.00+0

Starvation- and Stationary-Phase-Induced Acid Tolerance in *Escherichia coli* O157:H7

K. W. ARNOLD AND C. W. KASPAR*

Department of Food Microbiology and Toxicology, Food Research Institute, University of Wisconsin, Madison, Wisconsin 53706-1187

Received 1 December 1994/Accepted 9 March 1995

Stationary phase and the starvation of log-phase cells increased the acid tolerance of *Escherichia coli* O157:H7 strains. Although the degree of acid tolerance varied, the survival of most O157:H7 strains exceeded that of other, related, pathogens in a synthetic gastric fluid.

Escherichia coli O157:H7 is recognized as an important cause of hemorrhagic colitis and hemolytic uremic syndrome that is disseminated by foods, water, and person-to-person contact (13). Although ground beef has been implicated as the primary vehicle of transmission (13), outbreaks of *E. coli* O157:H7 involving apple cider (2), mayonnaise (27), and yogurt (24) have raised concerns about the safety of high-acid foods and the acid tolerance properties of this pathogen. In addition to epidemiological data, studies of survival in acidic foods (1, 2, 10, 23, 25, 27) demonstrate that *E. coli* O157:H7 is acid tolerant, particularly at lower temperatures (23, 27).

Studies with nonpathogenic *E. coli* and *Salmonella typhimurium* demonstrate that prior exposure to acidic conditions or growth in a moderately acidic medium enhances survival at low pH (7, 8, 11). This inducible acid tolerance system promotes survival of *S. typhimurium* in cheese (20) and is important to the virulence of the organism (9). Recently, two pH-dependent systems were described; one is induced in log phase, and the other is induced in stationary phase (7, 19, 26). A third system, which is pH independent, is induced by stationary phase and involves the *rpoS* gene encoding sigma factor σ^{38} (14, 18, 19, 21, 26). The present study was initiated to identify environmental and physiological factors affecting acid tolerance in *E. coli* O157:H7 and to compare the acid tolerance of O157:H7 strains with that of other, related, pathogens.

Seven strains of *E. coli* O157:H7 (ATCC 43889, ATCC 43894, ATCC 43895, 84-01, TB 226, TB 285, and JBL 1935); enteroinvasive *E. coli* serotypes O124 and O152 (10011 and 10010, respectively); enteropathogenic *E. coli* serotypes O111:H2 and O127:H6 (B170 and E2348169, respectively); a nonpathogenic control strain, *E. coli* C (Food Research Institute—Kaspar [FRIK] culture collection isolate 123); and *Shigella dysenteriae* type 1 (ATCC 29026) were used in these studies. Stock cultures were maintained in liquid nitrogen in 10% glycerol. Cultures were grown overnight in Trypticase soy broth (TSB) (BBL Microbiology Systems, Cockeysville, Md.) at 35°C with shaking (150 rpm) and transferred to fresh medium on consecutive days at least twice before use in experiments. Cultures in the logarithmic and stationary phases of growth were obtained by monitoring growth at 35°C in TSB spectrophotometrically: mid-log phase, $A_{600} = 0.3$ (1.2 h, ca. 1×10^8 CFU/ml); late log phase, $A_{600} = 0.7$ (3 h, ca. 3×10^8 CFU/ml); and stationary phase, $A_{600} = 1.1$ (7 h, ca. 1×10^9 CFU/ml).

The number of viable cells in samples was determined by plating on Trypticase soy agar (Difco Laboratories, Detroit, Mich.) either directly or following dilution in 0.1% peptone. The numbers of CFU per milliliter were determined from duplicate plates after incubation at 35°C for 20 to 24 h; longer incubation times did not yield higher numbers of CFU.

The acid tolerance of strains was assessed in TSB adjusted to pH 2 with 5 N HCl. Cultures diluted in 0.1% peptone were added to duplicate flasks containing 100 ml of acidified TSB (pH 2) at a final concentration of ca. 10^4 CFU/ml. The number of CFU per milliliter of the inoculum was also determined so that the number of CFU added to the assay flask could be calculated. Following inoculation, the numbers of CFU per milliliter were determined immediately (ca. 1 min; time zero [T_0]) and again after incubation for 4 h at 4 or 25°C. Because the number of CFU per milliliter decreased, under some conditions, during the time of inoculation and sample removal (ca. 1 min), the number of CFU at T_0 was determined from the inoculum CFU per milliliter and the quantity added to the acid challenge flask. The percent log survivors was calculated as follows: (log CFU per milliliter at T_4 divided by the log CFU per milliliter at T_0) $\times$ 100.

In starvation studies, *E. coli* O157:H7 (ATCC 43895) was grown to mid-log phase and the cells were harvested by centrifugation (8,820 $\times$ *g*, 5 min), washed, resuspended in phosphate-buffered saline (PBS) (0.01 M, pH 7.0), and then added to duplicate flasks containing 250 ml of sterile PBS. Flasks were inoculated to achieve a final concentration of ca. 10^5 CFU/ml and incubated at 4 or 25°C. Chloramphenicol (Sigma Chemical Co., St. Louis, Mo.) was added to a duplicate set of flasks at a final concentration of 10 μg/ml. Samples were removed after 6, 24, and 48 h of incubation, and the cells were tested for acid tolerance at 25°C.

A synthetic gastric fluid was prepared essentially as described by Beumer et al. (4), except that bovine bile (Sigma) was used in place of porcine bile. Compounds were dissolved in deionized water, adjusted to pH 1.5 with HCl (5 N), and filter sterilized (Nalgene polycarbonate membrane; 0.45-μm pore size). Stationary-phase cultures were used to inoculate each of three flasks containing 100 ml of prewarmed (37°C) synthetic gastric fluid at a final concentration of ca. 10^4 CFU/ml. Survival was monitored periodically during incubation at 37°C with shaking (150 rpm).

The data from growth phase experiments were examined by a split-plot analysis of variance, whereas data on survival in synthetic gastric fluid were compared by a one-way analysis of variance. Data are the average values from two or more flasks

* Corresponding author. Mailing address: Food Research Institute, 1925 Willow Drive, Madison, WI 53706. Phone: (608) 263-6936. Fax: (608) 263-1114. Electronic mail address: CWKaspar@facstaff.wisc.edu.

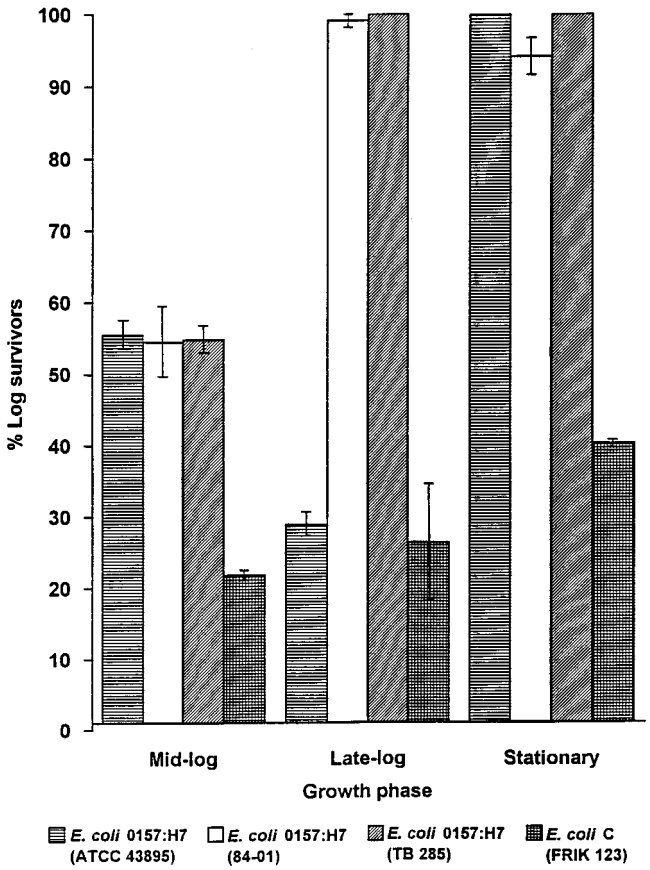

FIG. 1. Survival of three *E. coli* O157:H7 strains (ATCC 43895, 84-01, and TB 285) and an *E. coli* control strain (FRIK 123) in acidified TSB (pH 2) when tested at mid-log, late log, and stationary phases of growth. Error bars represent the standard deviation of the means.

and were analyzed with the generalized linear models module of the Statistical Analysis System (SAS Institute, Inc., Cary, N.C.).

The acid tolerance of *E. coli* O157:H7 was not dependent on prior exposure to a low pH. When the *E. coli* O157:H7 strain ATCC 43895 was grown overnight (final pH 7.2) in Luria broth, 79% of the cells survived acid challenge whereas <0.001% of the *E. coli* control strain cells (*E. coli* C, isolate FRIK 123) grown under the same conditions survived (data not shown). Thus, the mechanism(s) of acid tolerance in *E. coli* O157:H7 differs from or complements the acid tolerance response described in *S. typhimurium*, in which cells exposed to or grown in a moderately acidic medium become more tolerant of low-pH conditions (7, 8, 19).

To determine if acid tolerance in *E. coli* O157:H7 is growth phase dependent, as in *rpoS*-regulated systems (12, 26), the survival of three O157:H7 strains (84-01, TB 285, and ATCC 43895) and an *E. coli* control strain (FRIK 123) was examined with mid-log-, late-log-, and stationary-phase cells challenged in acidified TSB (pH 2). Results (Fig. 1) show that mid-log-phase cultures of O157:H7 strains and the control strain were acid sensitive, because numbers declined approximately 100-fold or more during acid challenge. In late-log-phase cultures, two O157:H7 strains (84-01 and TB 285) were more tolerant to acid challenge (99 and 100% log survivors, respectively). *E. coli* O157:H7 strain ATCC 43895 was more acid tolerant (100% log survivors) in stationary phase, whereas the tolerance of O157:H7 strains 84-01 and TB 285 remained unchanged. The *E. coli* control strain (FRIK 123) was significantly less tolerant

TABLE 1. Effects of starvation, temperature, and chloramphenicol on the development of acid tolerance in mid-log-phase *E. coli* O157:H7 (ATCC 43895)

Temp and sample time[a]	Log CFU/ml after acid challenge (pH 2.0)		% Log survivors[c]
	T_0[b]	T_4	
4°C			
6 h	4.1	1.0	24.1
24 h	3.0	ND[d]	
48 h	2.1	ND	
25°C			
6 h	4.4	2.7	61.4
24 h	4.3	3.6	83.7
48 h	4.1	3.2	78.0
25°C + CM[e]			
6 h	4.3	1.9	44.2
24 h	4.3	2.1	48.8
48 h	3.8	2.2	57.9

[a] Cells were incubated in PBS with shaking (75 rpm).
[b] Log CFU per milliliter added to acid challenge flask.
[c] Calculated as (log CFU per milliliter after acid challenge [T_4] divided by log CFU per milliliter [T_0]) × 100.
[d] ND, none detected (<10 CFU/ml).
[e] CM, chloramphenicol (10 μg/ml).

($P < 0.0001$) than the O157:H7 strains, although the numbers of survivors did increase between mid-log (22% log survivors) and stationary (40% log survivors) phases. The induction of acid tolerance in *E. coli* O157:H7 during late log and stationary phases is consistent with studies demonstrating enhanced resistance to a variety of chemical and physical challenges in stationary-phase cultures of *E. coli* and *Streptococcus faecalis* (3, 14, 17) and *rpoS*-regulated properties (14, 18, 21).

To determine if environmental conditions, i.e., nutrient levels and temperature, induce or influence acid tolerance, mid-log-phase *E. coli* O157:H7 strain ATCC 43895 was added to flasks of PBS and incubated at 4 or 25°C. Starvation at 4°C was detrimental to strain ATCC 43895 survival, as viable counts decreased 100-fold by 48 h. Additionally, the surviving cells were acid sensitive, as viable counts decreased >10- to 100-fold during acid challenge (Table 1). In contrast, cells starved at 25°C for 24 and 48 h were more acid tolerant (83.7 and 78.0% log survivors, respectively). Because protein synthesis in *E. coli* is not likely to occur at temperatures below 8°C (5), incubation at 4°C may have inhibited the production of a protein(s) necessary for acid tolerance. The need for protein synthesis in starvation-induced acid tolerance was demonstrated by the addition of chloramphenicol (10 μg/ml) to PBS cell suspensions, which resulted in cells that were significantly ($P < 0.0001$) less acid tolerant than cells starved without chloramphenicol. These findings are in agreement with studies that demonstrated that starvation-induced proteins protect *E. coli* against both chemical and physical challenges (14–17). Additional studies are needed to determine whether stationary-phase- and starvation-induced acid tolerances in *E. coli* O157:H7 involve a common protein(s) and/or regulator.

The survival of stationary-phase cultures of *E. coli* O157:H7 was compared with that of two enteroinvasive *E. coli* serotypes (O124 and O152), two enteropathogenic *E. coli* serotypes (O111:H2 and O127:H6), an *E. coli* control strain (FRIK 123), and *S. dysenteriae* (ATCC 29026) in synthetic gastric fluid (4) (pH 1.5) (Table 2). Survival in synthetic gastric fluid, instead of in acidified TSB, was examined to determine if the acid tolerance of *E. coli* O157:H7 may be sufficient to protect it from the acidic conditions of the stomach. All five *E. coli* O157:H7

TABLE 2. Survival of *E. coli* O157:H7, enteroinvasive *E. coli*, enteropathogenic *E. coli*, *E. coli* C (control strain), and *S. dysenteriae* in synthetic gastric fluid (pH 1.5)

Strain	% Log survivors[a] at *T* (h):					
	0.0	0.5	1.0	1.5	2.0	3.0
E. coli O157:H7						
ATCC 43889	95.7	58.7	21.7	ND[b]	ND	ND
ATCC 43894	100	93.0	79.1	65.1	53.5	30.2
ATCC 43895	100	93.0	79.1	67.4	53.5	39.5
TB 226	100	92.7	85.4	78.0	73.2	61.0
JBL 1935	97.7	93.2	79.5	70.5	63.6	43.2
Other *E. coli* serotypes						
Enteroinvasive, O124	90.7	58.1	37.2	23.3	ND	ND
Enteroinvasive, O152	95.3	55.8	32.6	23.3	ND	ND
Enteropathogenic, O111:H2	90.7	44.2	ND	ND	ND	ND
Enteropathogenic, O127:H6	95.1	90.2	85.4	82.9	73.2	53.7
E. coli C (control), FRIK 123	92.7	29.3	ND	ND	ND	ND
S. dysenteriae type 1, ATCC 29026	63.4	ND	ND	ND	ND	ND

[a] Percent based on CFU per milliliter added to synthetic gastric fluid.
[b] ND, none detected (<10 CFU/ml).

strains survived 1 h in pH 1.5 gastric fluid, and four strains were still detectable after 3 h of incubation. Strain TB 226 was the most tolerant O157:H7 strain, as the numbers decreased at the slowest rate and this strain was present at the highest level after 3 h of incubation (61% log survivors). A strain of enteropathogenic *E. coli*, serotype O127:H6, also survived 3 h of incubation (53.7% log survivors). The survival of TB 226 and of the O127:H6 strain did not significantly differ, but both strains were significantly more acid tolerant than the remaining strains examined ($P = 0.005$). In contrast, *E. coli* O157:H7 strain ATCC 43889 had one of the shortest survival times and was undetectable after 1 h of incubation. The strain-to-strain variation in acid tolerance of *E. coli* O157:H7 strains has been reported previously (6, 23). The remaining pathogenic (serotypes O124, O111:H2, and O152) and control *E. coli* (FRIK 123) strains were undetectable after ≤2 h of incubation in gastric fluid. *S. dysenteriae* was not detectable after 0.5 h of incubation, which was unexpected since *S. flexneri* has been reported to be acid tolerant (12). Considering that the clearance time of an average meal from the stomach is around 3 h (22) and the stomach pH is usually higher when food is present, the acid tolerance of *E. coli* O157:H7 may be important to its pathogenesis.

The findings that stationary-phase and starved *E. coli* O157:H7 strains are more acid tolerant than mid-log-phase cells are consistent with previous studies of stationary-phase cells, starved cells (3, 14–16), and *rpoS*-regulated properties (12, 14, 17). However, the mechanism and/or the level of protective protein(s) produced in *E. coli* O157:H7 must differ because the degree of acid tolerance in some O157:H7 strains is greater than that in other bacteria that also contain starvation-induced and *rpoS*-regulated systems (12, 14, 26). It is possible that multiple protective systems are active simultaneously in *E. coli* O157:H7. With a greater understanding of the regulation of acid tolerance in *E. coli* O157:H7, it may be possible to devise control strategies for this pathogen by regulating environmental conditions that trigger the production of protective proteins.

We thank Jim Kaper, Keith Lampel, John Luchansky, and Phil Tarr for providing strains; Alejandro Muñoz-del-Río for assistance with statistical analysis; Jeffrey Farber for insightful discussions; and Leslie Garland Miller for technical assistance.

This project was supported by grant 94-37201-1025 from the National Research Initiative Competitive Grants Program of the U.S. Department of Agriculture; by the College of Agriculture and Life Sciences, University of Wisconsin—Madison; and by contributions to the Food Research Institute.

REFERENCES

1. **Abdul-Raouf, U. M., L. R. Beuchat, and M. S. Ammar.** 1993. Survival and growth of *Escherichia coli* O157:H7 in ground, roasted beef as affected by pH, acidulants, and temperature. Appl. Environ. Microbiol. **59:**2364–2368.
2. **Besser, R. E., S. M. Lett, J. T. Weber, M. P. Doyle, T. J. Barrett, J. G. Wells, and P. M. Griffin.** 1993. An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157:H7 in fresh-pressed apple cider. JAMA **269:**2217–2220.
3. **Beuchat, L. R., and R. V. Lechowich.** 1968. Survival of heated *Streptococcus faecalis* as affected by phase of growth and incubation temperature after thermal exposure. J. Appl. Bacteriol. **31:**414–419.
4. **Beumer, R. R., J. de Vries, and F. M. Rombouts.** 1992. *Campylobacter jejuni* nonculturable coccoid cells. Int. J. Food Microbiol. **15:**153–163.
5. **Broeze, R. J., C. J. Solomon, and D. H. Pope.** 1978. Effects of low temperature on in vivo and in vitro protein synthesis in *Escherichia coli* and *Pseudomonas fluorescens*. J. Bacteriol. **134:**861–874.
6. **Cutter, C. N., and G. R. Siragusa.** 1994. Efficacy of organic acids against *Escherichia coli* O157:H7 attached to beef carcass tissue using a pilot scale model carcass washer. J. Food Prot. **57:**97–103.
7. **Foster, J. W.** 1993. The acid tolerance response of *Salmonella typhimurium* involves transient synthesis of key acid shock proteins. J. Bacteriol. **175:** 1981–1987.
8. **Foster, J. W., and H. K. Hall.** 1991. Inducible pH homeostasis and the acid tolerance response of *Salmonella typhimurium*. J. Bacteriol. **173:**5129–5135.
9. **Gardia-del Portillo, F., J. W. Foster, and B. B. Finlay.** 1993. Role of acid tolerance response genes in *Salmonella typhimurium* virulence. Infect. Immun. **61:**4489–4492.
10. **Glass, K. A., J. M. Loeffelholz, J. P. Ford, and M. P. Doyle.** 1992. Fate of *Escherichia coli* O157:H7 as affected by pH or sodium chloride and in fermented, dry sausage. Appl. Environ. Microbiol. **58:**2513–2516.
11. **Goodson, M., and R. J. Rowbury.** 1989. Habituation to normal lethal acidity by prior growth of *Escherichia coli* at a sublethal acid pH value. Lett. Appl. Microbiol. **8:**77–79.
12. **Gorden, J., and P. L. C. Small.** 1993. Acid resistance in enteric bacteria. Infect. Immun. **61:**364–367.
13. **Griffin, P. M., and R. V. Tauxe.** 1991. The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. Epidemiol. Rev. **13:**60–97.
14. **Hengge-Aronis, R.** 1993. Survival of hunger and stress: the role of *rpo*S in early stationary phase gene regulation in *E. coli*. Cell **72:**165–168.
15. **Jenkins, D. E., S. A. Chaisson, and A. Matin.** 1990. Starvation-induced cross protection against osmotic challenge in *Escherichia coli*. J. Bacteriol. **172:** 2779–2781.
16. **Jenkins, D. E., J. E. Schultz, and A. Matin.** 1988. Starvation-induced cross protection against heat or H_2O_2 challenge in *Escherichia coli*. J. Bacteriol. **170:**3910–3914.
17. **Kolter, R., D. A. Siegele, and A. Tormo.** 1993. The stationary phase of the bacterial life cycle. Annu. Rev. Microbiol. **47:**855–874.
18. **Lange, R., and R. Hengge-Aronis.** 1991. Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. Mol. Microbiol. **5:**49–59.
19. **Lee, I. S., J. L. Slonczewski, and J. W. Foster.** 1994. A low-pH-inducible, stationary-phase acid tolerance response in *Salmonella typhimurium*. J. Bacteriol. **176:**1422–1426.
20. **Leyer, G. J., and E. A. Johnson.** 1993. Acid adaptation promotes survival of *Salmonella* spp. in cheese. Appl. Environ. Microbiol. **58:**2075–2080.
21. **McCann, M. P., J. P. Kidwell, and A. Matin.** 1991. The putative σ factor KatF has a central role in development of starvation-mediated general resistance in *Escherichia coli*. J. Bacteriol. **173:**4188–4194.
22. **McMinn, R. M. H., and M. H. Hobdell.** 1974. Stomach, p. 127–150. *In* Functional anatomy of the digestive system. The Pitman Press, Bath, United Kingdom.
23. **Miller, L. G., and C. W. Kaspar.** 1994. *Escherichia coli* O157:H7 acid tolerance and survival in apple cider. J. Food Prot. **57:**460–464.
24. **Morgan, D., C. P. Newman, D. N. Hutchinson, A. M. Walker, B. Rowe, and F. Majid.** 1993. Verotoxin producing *Escherichia coli* O157 infections associated with the consumption of yoghurt. Epidemiol. Infect. **111:**181–187.
25. **Reitsma, C. J., and D. R. Henning.** 1994. Survival of *Escherichia coli* O157:H7 during the manufacture and curing of cheddar cheese. J. Dairy Sci. **77**(Suppl. 1)**:**30.
26. **Small, P., D. Blankenhorn, D. Welty, E. Zinser, and J. L. Slonczewski.** 1994. Acid and base resistance in *Escherichia coli* and *Shigella flexneri*: role of *rpoS* and growth pH. J. Bacteriol. **176:**1729–1737.
27. **Weagant, S. D., J. L. Bryant, and D. H. Bark.** 1994. Survival of *Escherichia coli* O157:H7 in mayonnaise and mayonnaise-based sauces at room and refrigerated temperatures. J. Food Prot. **57:**629–631.