

rpoS Regulation of Acid, Heat, and Salt Tolerance in *Escherichia coli* O157:H7

A. M. CHEVILLE, K. W. ARNOLD, C. BUCHRIESER, C.-M. CHENG, AND C. W. KASPAR*

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

Received 11 December 1995/Accepted 9 March 1996

An *rpoS* mutant (*rpoS*::pRR10) of *Escherichia coli* O157:H7 ATCC 43895 was generated. Stationary-phase acid, heat, and salt tolerance was significantly reduced, and starvation-induced acid tolerance did not develop in the mutant. RpoS was also important for survival of *E. coli* O157:H7 in dry, fermented sausage.

Since the first recognized outbreak in 1982, *Escherichia coli* O157:H7 has emerged as a food-borne pathogen of significant public health concern (13). Outbreaks involving acidic foods (i.e., apple cider; dry, fermented sausage; mayonnaise; and yoghurt) (4, 5, 23, 29) have drawn attention to the acid tolerance properties of *E. coli* O157:H7 (2, 3, 6, 22). In addition to promoting survival in foods, acid tolerance may play a role in *E. coli* O157:H7 pathogenesis by promoting survival during passage through the stomach (2, 3, 12).

Stationary phase (2, 18) and starvation (2, 10, 16, 17, 24–26) induce protective proteins that impart resistance to chemical and physical challenges. Entrance into stationary phase results in the production of 30 proteins in *E. coli* which are regulated by σ^s (*rpoS*) (14, 15, 21). Studies of nonpathogenic *E. coli* have associated stationary-phase resistance properties with *rpoS*-regulated proteins (10, 28); however, it is not known if a similar or modified system is responsible for acid tolerance and other resistance properties in serotype O157:H7. Although acid-inducible systems are present (11, 28), sustained acid tolerance in *E. coli* O157:H7 is not dependent upon adaptation but is induced by stationary phase or starvation (2, 22). Thus, sustained acid tolerance in *E. coli* O157:H7 may be part of a general protective system triggered by stationary phase rather than a specific acid tolerance system.

In this study, an *rpoS* mutant of *E. coli* O157:H7 was generated to define the role of *rpoS*-regulated proteins in acid, heat, and salt tolerance, as well as survival in dry, fermented sausage.

E. coli S17-1, provided by F. Fang (9) and containing a mobilizable suicide plasmid with a 600-bp *rpoS* fragment (*rpoS*::pRR10) from *Salmonella typhimurium* and the gene for β -lactamase, was used to generate an *rpoS* mutant of *E. coli* O157:H7 (ATCC 43895). *E. coli* S17-1 was grown in Luria-Bertani (LB) broth (19) containing 250 μ g of penicillin (pen) per ml (Sigma Chemical Company, St. Louis, Mo.). Strain ATCC 43895 was grown overnight in Trypticase soy broth (TSB) at 37°C with shaking (150 rpm) and transferred to fresh TSB on 2 consecutive days before use in survival studies. Stationary-phase cells (15 h; A_{600} , 1.2; ca. 10^9 CFU/ml) were used unless otherwise stated. *rpoS* mutants were grown similarly, except that TSB was supplemented with pen. Conjugation was performed by the procedure of Marugg et al. (20) with the following modifications. Cultures were grown overnight, di-

luted in LB broth (S17-1, 1:100; ATCC 43895, 1:10), and incubated for 1 h at 37°C with shaking (150 rpm) (27). A portion of each culture was mixed 1:1 (0.5 ml), pelleted ($10,000 \times g$, 30 s), resuspended in 50 μ l of LB broth, spread on a sterile 0.45- μ m-pore-size filter (mixed cellulose ester; Micron Separations, Inc., Westboro, Mass.), and placed on an LB agar plate. After incubation, conjugants were removed, resuspended in 10 ml of LB broth, and spread plated (0.1-ml portions) onto oxidative fermentative medium (Difco) containing 1% sucrose (Difco) and 250 μ g of pen per ml. After 24 h of incubation at 37°C, pen-resistant, sucrose-positive (yellow) colonies were selected and restreaked on oxidative fermentative medium-pen plates (ATCC 43895, Suc⁺; *E. coli* S17-1, Suc⁻). Colonies were tested for the O157 antigen by agglutination (Oxoid, Basingstoke, England).

Mutants were confirmed by the procedure of Fang et al. (9), except that a digoxigenin-labeled *rpoS* fragment was used in hybridizations (Genius 1 DNA Labeling and Detection Kit; Boehringer Mannheim, Indianapolis, Ind.). In addition, the specific activity (units per minute per milligram of protein) of acid phosphatase was determined as described by Dassa et al. (7).

In acid, heat, and salt challenges, cultures were diluted in 0.1% peptone and inoculated into challenge flasks (ca. 10^4 to 10^5 CFU/ml) containing 100 ml of synthetic gastric fluid (pH 1.5) (2), acidified TSB (pH 2), TSB with 2.5 M NaCl (15%), or prewarmed TSB (55°C). Acid tolerance was assessed at 4 and 25°C in TSB and at 37°C in synthetic gastric fluid. Salt tolerance was assessed at 25°C. Studies of starvation-induced acid tolerance were conducted as previously described (2), with log-phase cells (A_{600} , 0.3; ca. 1.0×10^8 CFU/ml) at 25°C. CFU were enumerated following incubation on tryptic soy agar (Difco) at 37°C for 20 h and up to 48 h.

Survival of *E. coli* O157:H7 on retail fermented, cooked sausages (26.8% fat, 1.2% salt) was also monitored by using 10-g slices inoculated with 0.1 ml of culture at a final concentration of ca. 10^4 CFU/g. Slices were stored at 4°C in individual Whirl-Pak bags (Nasco, Fort Atkinson, Wis.) for 21 days. To determine numbers of *E. coli* O157:H7, samples (slices) were periodically removed, diluted 1:10 in 0.1% peptone, homogenized (1 min; Stomacher 400; Tekmar, Inc., Cincinnati, Ohio), spread plated on tryptic soy agar, and enumerated as described above. Randomly selected colonies (five per plate) were confirmed for the O157 serotype by agglutination (Oxoid). Uninoculated control slices were tested on days 0 and 21 for O157-positive colonies.

The mean number of CFU per milliliter was determined from duplicate plate counts and three trials in all experiments.

* Corresponding author. Mailing address: Food Research Institute, 1925 Willow Drive, Madison, WI 53706-1187. Phone: (608) 263-6936. Fax: (608) 263-1114. Electronic mail address: cwkaspar@facstaff.wisc.edu.

TABLE 1. Stationary-phase- and starvation-induced acid tolerance of *E. coli* O157:H7 (ATCC 43895) and *rpoS* mutant FRIK 816-3

Strain	Acid tolerance of stationary-phase cells (avg <i>D</i> value [h] ± SE)			Acid tolerance (avg % log survivors ± SE) of log-phase cells starved in PBS ^a for:				
	SGF ^b 37°C	TSB ^c 4°C	TSB 25°C	0 h	6 h	24 h	48 h	72 h
ATCC 43895	1.0 ± 0.1 ^d	60.9 ± 7.4	56.2 ± 1.7	ND ^e	ND	86.0 ± 0.1	77.5 ± 0.1	82.8 ± 0.0
FRIK 816-3	0.4 ± 0.0	1.7 ± 0.2	9.1 ± 0.2	ND	ND	ND	ND	ND

^a Cells were challenged in TSB (pH 2.0) for 24 h at 25°C. PBS, phosphate-buffered saline.

^b SGF, synthetic gastric fluid at pH 1.5.

^c TSB at pH 2.0.

^d Standard error from three separate trials; duplicate samples were analyzed at each time point.

^e ND, none detected (<10 CFU/ml).

Analysis of variance was conducted by using the Statistical Analysis System (SAS Institute, Inc., Cary, N.C.).

Homologous recombination and insertion of *rpoS*::pRR10 into the *rpoS* gene of strain ATCC 43895 was confirmed by Southern hybridization. All *rpoS* mutants (FRIK 816-2, FRIK 816-3, FRIK 816-6, and FRIK 816-10) produced identical hybridization patterns (data not shown). Insertion of pRR10 into *rpoS* was also demonstrated phenotypically by measuring acid phosphatase, which is *rpoS* regulated (15). The specific activity of acid phosphatase (units per minute per milligram of protein) was significantly less ($P < 0.0001$) in the *rpoS* mutants (63.4 ± 9.5 to 86.0 ± 14.8) than in wild-type strain ATCC 43895 (193.6 ± 19.9). Residual activity detected in the mutants was likely due to *rpoS*-independent regulation of acid phosphatase (14).

All four *rpoS* mutants were significantly less ($P < 0.0001$) acid tolerant (23.1 to 47.6% log survivors) than strain ATCC 43895 (100% log survivors) after 4 h in acidified TSB (pH 2) at 4°C (data not shown). FRIK 816-3 was used in the remaining studies. *D* values (time for a 1-log reduction) of FRIK 816-3 (0.4 ± 0.0 h) and ATCC 43895 (1.0 ± 0.1 h) in synthetic gastric fluid (pH 1.5) indicate that *rpoS*-regulated proteins are important to *E. coli* O157:H7 survival in acidic environments (Table 1). Temperature also influenced the acid tolerance (TSB, pH 2) of FRIK 816-3, as *D* values were five times higher at 25°C (9.1 ± 0.2 h) than at 4°C (1.7 ± 0.2 h), whereas the acid tolerance of strain ATCC 43895 was not significantly different at the two temperatures (56.2 ± 1.7 h at 25°C and 60.9 ± 7.4 h at 4°C) (Table 1).

Starvation of log-phase FRIK 816-3 cells did not induce acid tolerance, whereas strain ATCC 43895 developed acid tolerance after ≥ 24 h of starvation in phosphate-buffered saline (Table 1), suggesting that *rpoS*-regulated proteins are also responsible for starvation-induced acid tolerance in *E. coli* O157:

H7. When starved cells were acid challenged for 48 h (TSB, pH 2), the number of survivors increased with longer periods of starvation (data not shown). A comparison of starvation- and stationary-phase-induced acid tolerance in strain ATCC 43895 found that starvation conferred significantly lower levels ($P < 0.0001$) of acid tolerance (TSB, pH 2, 24 h) than did stationary phase, although longer periods of starvation (72 h) minimized the difference, 82.8 and 93.8% log survivors, respectively. These results indicate that stationary phase may induce protective proteins that are lacking or produced more slowly or at lower levels in starved *E. coli* O157:H7. In addition, acid-adapted (growth at pH 5) mid- and late-log-phase cells of strain ATCC 43895 were significantly less ($P < 0.0001$) acid tolerant than starved or stationary-phase cultures (data not shown), demonstrating that *rpoS*-regulated proteins impart sustained acid tolerance to *E. coli* O157:H7.

Strain FRIK 816-3 was also less heat and salt tolerant than strain ATCC 43895 (Fig. 1A and B). After 7 min at 55°C, strain FRIK 816-3 decreased >4 logs and was at the minimum detection level ($39.9\% \pm 3.9\%$ log survivors), whereas $63.1\% \pm 4.0\%$ log survivors of strain ATCC 43895 were present after 28 min. Although *E. coli* O157:H7 is not unusually heat tolerant (1, 8), these data demonstrate that in the absence of *rpoS*-regulated proteins heat sensitivity is significantly increased. Likewise, in 2.5 M NaCl, the percent log survivors after 24 h were ca. $51.1\% \pm 3.0\%$ and $93.8\% \pm 0.7\%$ for FRIK 816-3 and ATCC 43895, respectively.

Because *rpoS*-regulated proteins enhanced tolerance to a variety of chemical and physical challenges, it was not surprising that the *rpoS* mutant survived poorly in dry, fermented sausage (pH 4.6 to 4.8, 1.2% salt, 1.9% moisture). As shown in Fig. 1C, numbers of FRIK 816-3 cells were 2.3 logs lower than those of ATCC 43895 cells after 21 days. Background flora was not detected in uninoculated control slices. All selected colonies tested positive for the O157 antigen.

Our findings underscore the significance of RpoS for sustained acid tolerance in *E. coli* O157:H7. Furthermore, *rpoS*-regulated proteins cross-protect against heat and salt challenges and promote survival of O157:H7 in fermented sausage. Additional studies on the identification and function of the *rpoS*-regulated proteins involved in general stress protection are warranted. In addition, the identification of physiochemical conditions that trigger synthesis of protective *rpoS*-regulated proteins should be useful in devising control strategies for *E. coli* O157:H7 in foods.

We are grateful to Ferric Fang for supplying *rpoS*::pRR10 and to Kathy Becker for technical assistance.

This research was supported by grant 94-37201-1025 from the National Research Initiative Competitive Grants Program of the U.S. Department of Agriculture; S. C. Johnson Wax, Inc.; and the College of Agricultural and Life Sciences, University of Wisconsin-Madison.

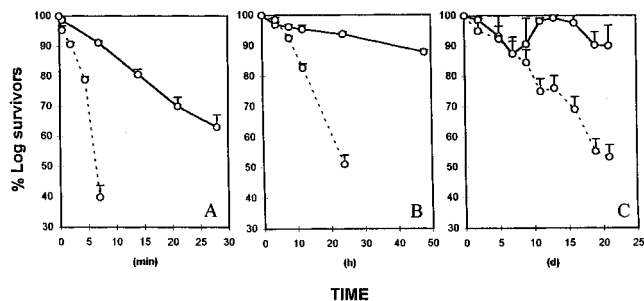


FIG. 1. Heat (A) and salt (B) tolerance and survival in dry, fermented sausage (C) of stationary-phase *E. coli* O157:H7 ATCC 43895 (○—○) and *rpoS* mutant FRIK 816-3 (○---○). Error bars represent standard deviations of the means.

REFERENCES

- Ahmed, N. M., D. E. Conner, and D. L. Huffman. 1995. Heat resistance of *Escherichia coli* O157:H7 in meat and poultry products as affected by product composition. *J. Food Sci.* **60**:606–610.
- Arnold, K. W., and C. W. Kaspar. 1995. Starvation- and stationary phase-induced acid tolerance in *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* **61**:2037–2039.
- Benjamin, M. M., and A. R. Datta. 1995. Acid tolerance of enterohemorrhagic *Escherichia coli*. *Appl. Environ. Microbiol.* **61**:1669–1672.
- 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.
- Centers for Disease Control and Prevention. 1995. Preliminary report: *Escherichia coli* O157:H7 outbreak linked to commercially distributed dry-cured salami—Washington and California, 1994. *Morbidity and Mortality Weekly Report* **44**:157–160.
- Conner, D. E., and J. S. Kotrola. 1994. Growth and survival of *Escherichia coli* O157:H7 under acidic conditions. *Appl. Environ. Microbiol.* **61**:382–385.
- Dassa, J., H. Fsihi, C. Marck, M. Dion, M. Kieffer-Bontemps, and P. L. Boquet. 1992. A new oxygen-regulated operon in *Escherichia coli* comprises the genes for a putative third cytochrome oxidase and for pH 2.5 acid phosphatase (*appA*). *Mol. Gen. Genet.* **229**:342–352.
- Doyle, M. P. 1991. *Escherichia coli* O157:H7 and its significance in foods. *Int. J. Food Microbiol.* **12**:289–302.
- Fang, F. C., S. J. Libby, N. A. Buchmeier, P. C. Loewen, J. Switala, J. Harwood, and D. G. Guiney. 1992. The alternative σ factor KatF (RpoS) regulates *Salmonella* virulence. *Proc. Natl. Acad. Sci. USA* **89**:11978–11982.
- Gauthier, M. J., and R. L. Clement. 1994. Effect of a short period of starvation in oligotrophic waters on the resistance of enteric bacterial pathogens to gastric pH conditions. *FEMS Microbiol. Ecol.* **14**:275–284.
- Goodson, M., and R. J. Rowbury. 1989. Habituation to normally lethal acidity by prior growth of *Escherichia coli* at a sub-lethal acid pH value. *Lett. Appl. Microbiol.* **8**:77–79.
- Gorden, J., and P. L. C. Small. 1993. Acid resistance in enteric bacteria. *Infect. Immun.* **61**:364–367.
- 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.
- Hengge-Aronis, R. 1993. The role of *rpoS* in early stationary phase gene regulation in *Escherichia coli* K12, p. 171–194. *In* S. Kjelleberg (ed.), *Starvation in bacteria*. Plenum Press, New York.
- Hengge-Aronis, R. 1993. Survival of hunger and stress: the role of *rpoS* in early stationary phase gene regulation in *E. coli*. *Cell* **72**:165–168.
- 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.
- Jenkins, D. E., J. E. Schultz, and A. Matin. 1988. Starvation-induced cross protection against heat or H₂O₂ challenge in *Escherichia coli*. *J. Bacteriol.* **170**:3910–3914.
- 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.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Marugg, J. D., M. Van Spanje, W. P. M. Hoekstra, B. Schippers, and P. J. Weisbeek. 1985. Isolation and analysis of genes involved in siderophore biosynthesis in plant-growth-stimulating *Pseudomonas putida* WCS358. *J. Bacteriol.* **164**:563–570.
- 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.
- 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. [Erratum, **57**:645.]
- 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.
- Morton, D. S., and J. D. Oliver. 1994. Induction of carbon starvation-induced proteins in *Vibrio vulnificus*. *Appl. Environ. Microbiol.* **60**:3653–3659.
- Munro, P. M., G. N. Flatau, R. L. Clement, and M. J. Gauthier. 1995. Influence of the RpoS (KatF) sigma factor on maintenance of viability and culturability of *Escherichia coli* and *Salmonella typhimurium* in seawater. *Appl. Environ. Microbiol.* **61**:1853–1858.
- Reeve, C. A., P. S. Amy, and A. Matin. 1984. Role of protein synthesis in the survival of carbon-starved *Escherichia coli* K-12. *J. Bacteriol.* **160**:1041–1046.
- Simon, R., M. O'Connell, M. Labes, and A. Pühler. 1986. Plasmid vectors for the genetic analysis and manipulation of rhizobia and other gram-negative bacteria. *Methods Enzymol.* **118**:640–641.
- 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.
- Weagant, S. D., J. L. Bryant, and D. H. Bark. 1994. Survival of *Escherichia coli* O157:H7 in mayonnaise-based sauces at room and refrigerated temperatures. *J. Food Prot.* **57**:629–631.