APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1995, p. 1669–1672 Vol. 61, No. 4
0099-2240/95/$04.00+0

Acid Tolerance of Enterohemorrhagic *Escherichia coli*

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Received 2 September 1994/Accepted 7 February 1995

Enterohemorrhagic *Escherichia coli* (EHEC) strains were tested for their ability to survive in acid pH at 37°C. No loss of viability was observed in an O157:H7 EHEC strain (ATCC 43895) at pH levels of 3.0 and 2.5 for at least 5 h. The level of acid tolerance of most EHEC isolates was very high, similar to that of *Shigella flexneri* strains. The acid tolerance was dependent on the growth phase and pH of the growth medium.

Recent outbreaks and sporadic cases of diarrheal disease caused by enterohemorrhagic *Escherichia coli* (EHEC) have underscored the importance of this bacterium as an enteric pathogen. Besides diarrhea, EHEC causes hemorrhagic colitis and hemolytic-uremic syndrome—two potentially life-threatening conditions (13). Most EHEC infections are caused by contaminated food or water; however, they may also be acquired through human contact. The virulence properties of EHEC strains have been associated with the ability of the organism to attach to and efface intestinal mucosal cells and to elaborate two cytotoxins: Shiga-like toxin I and Shiga-like toxin II (14). These toxins, also known as verotoxins I and II, are analogous to Shiga toxin, with which they share a high degree of homology. Most EHEC strains isolated from humans with infections carry a large (65-MDa) plasmid which has been implicated in the adherence of the bacterium to intestinal mucosal cells (24). Although most episodes of hemorrhagic colitis and hemolytic-uremic syndrome are caused by EHEC strains that belong to serotype O157:H7, other serotypes have also been implicated (13).

The importance of gastric juice in controlling the outcome of food-borne infections is well recognized. To cause human illness, an invading organism must survive the acidic environment of the stomach before it reaches the intestine. Thus, the acidity of the gastric juice provides a first line of defense against food-borne pathogens. Reduction of gastric acidity has been associated with an increase in the survival rates of some common food-borne pathogens (20) and with a lowering of the infective dose (6, 22). Recently, Gorden and Small (12) showed that *Shigella* spp. are more acid tolerant (pH 2 to 2.5) than are *Salmonella* spp. and *E. coli*. They hypothesized that the high acid tolerance of *Shigella* spp. may contribute to their relatively low infective dose of 10 to 500 organisms. Benjamin and Datta (2) showed that strains of *Listeria monocytogenes* were more sensitive to a pH of 3.0 than were both *Shigella flexneri* and a laboratory strain of *E. coli*. The ability to survive in such a low pH depended on the growth phase for *Shigella* spp. (12) and the pH of the growth medium for *Listeria* spp. (16) and *Salmonella* spp. (10). The adaptive acid tolerance response was found to be controlled by several genes whose synthesis during adaptation was crucial for the response in *Salmonella* spp. (8, 9). Foster (9) showed that the adaptive acid tolerance response involves two distinct responses: prechallenge adaptation and transient adaptation, which occurs during low pH challenge.

EHEC strains isolated from various foods, including acidic foods, e.g., mayonnaise (15) and apple cider (3), survived and grew in vitro at pH 4.6 and above (1, 11). Miller and Kasper (19) found that the survival rates of two O157:H7 EHEC strains at pH levels both low and high (pH 12) were lower at 4°C than at 25°C. The present studies were undertaken to compare the survival rates of EHEC and *Shigella* strains in acidic (2.5 and 3.0) pH at 37°C and to evaluate the effect of the pH of the growth phase and growth medium on survival. These studies should be useful in evaluating the infective dose of this organism found in different foods stored under various environmental conditions.

To study the kinetics of acid sensitivity of EHEC strain AD305 (ATCC 43895), we routinely grew cultures in Luria broth (LB), pH 7.3, at 37°C. Fresh overnight cultures were diluted 1:1,000 in LB, adjusted to the desired pH with HCl, filter sterilized, and incubated at 37°C with gentle shaking. From the start of incubation (time 0), samples were periodically withdrawn, diluted appropriately with normal saline (0.85% NaCl in distilled water), and plated onto LB gelled with 1.5% Bacto agar (Difco Laboratories, Detroit, Mich.). The plates were incubated at 37°C, and CFU were counted after 18 h (Fig. 1). Strain AD305 (ATCC 43895) showed no loss of viability at pH levels of 3.0 and 2.5 for at least 5 h. However, the viability of *S. flexneri* AD324 decreased by 3.5 logs during the same period. The high level of acid tolerance in AD305 was in complete agreement with similar results obtained by Miller and Kasper (19) at 4 and 25°C. The acid tolerance of AD305 was not altered by keeping the culture stationary and thereby changing the aeration during the acid challenge. To determine whether the high acid tolerance of AD305 is strain or serotype (O157:H7) specific and if the EHEC strains are generally more acid tolerant, we tested the acid tolerance of several EHEC strains. For comparison, we also screened three more *S. flexneri* strains and one *L. monocytogenes* strain. The results indicated that not all of the EHEC strains tested were as acid tolerant as AD305, particularly at pH 2.5 (Table 1). Although these values (percent survival at pH 2.5) varied as much as 50% in the higher range of the values and by as much as two- to threefold at the lower range, it was possible (on the basis of three independent experiments) to tentatively group these strains into three categories: highly acid-tolerant (50 to 100% survival), moderately acid-tolerant (10 to 50% survival), and slightly acid-tolerant strains (<10% survival). The differences in survival rates at pH 2.5 among some strains (AD305 and AD317 compared with AD314 and AD319) appeared to be statistically significant; however, the real significance of these

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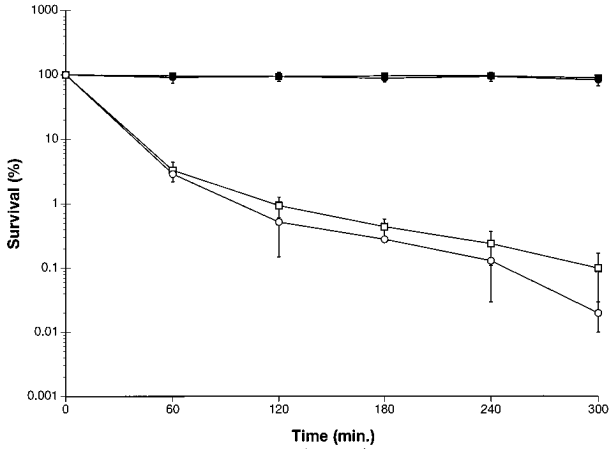

FIG. 1. Kinetics of acid tolerance of EHEC strain AD305 (●, ■) and *S. flexneri* AD324 (○, □) in LB acidified with HCl to pH 2.5 (○, ●) and pH 3.0 (□, ■). The points are averages of three independent experiments. The error bars are standard deviations.

values in terms of the in vivo survivability of the strains is not clear. No correlation was made with serotypes, the presence of one or both Shiga-like toxins, or the source of the isolates. *S. flexneri* strains exhibited a high to moderate level of acid tolerance, whereas the single *L. monocytogenes* strain tested was very sensitive. If a high level of acid tolerance in *Shigella* spp. is the possible cause of the low infective dose for shigellosis, as suggested by Gorden and Small (12), then the diseases caused by an EHEC strain like AD305 should also have a very low infective dose.

TABLE 1. Acid tolerance of bacterial strains

Strain	Serotype	Survival (%)[a]		Source or designation
		pH 2.5	pH 3.0	
E. coli				
AD305	O157:H7	100	100	ATCC 43895
AD314	O157:H7	0.1	28	ATCC 43894
AD316	O157:H7	10	26	ATCC 43888
AD317	O157:H7	72	80	ATCC 35150
AD318	O157:H7	17	32	ATCC 43889
AD319	O157:H7	0.65	44	ATCC 43890
AD320	O26:H	16	53	P. Tarr[b]
AD306	O26:H30	3	36	A. O'Brien[b]
AD307	O26:H11	26	74	N. Strockbine[b]
AD308	O111:NM	0.43	58	
AD313	O111:NM	0.38	35	
AD309	O22:H8	6	38	
AD310	O15:H27	20	40	
AD312	O165:H25	62	76	
S. flexneri				
AD321	5	98	111	K. Lampel[b]
AD322	5	92	79	
AD323	2a	12	10	
AD324	5	3	10	FDA collection
L. monocytogenes				
LS2	4b	0.34	18	FDA collection

[a] Fresh overnight cultures in LB, pH 7.3, were diluted 1:1,000 in LB adjusted with HCl to pH levels of 2.5 and 3.0. The cultures were incubated at 37°C for 2 h, and survival percentages were calculated from viable colonies.

[b] P. Tarr, Children's Hospital, Seattle, Wash.; A. O'Brien, Uniformed Services University of Health Sciences, Bethesda, Md.; N. Strockbine, Centers for Disease Control, Atlanta, Ga.; K. Lampel, Food and Drug Administration (FDA), Washington, D.C.

To understand the mechanism behind the high level of acid tolerance of AD305, we considered several possibilities. It is well documented that bacteria can survive and grow in a wide range of environmental pH; however, the mechanism by which they maintain internal pH in a narrow range (6.5 to 8.0) despite the large variations in outside pH is poorly understood (4). Several theories have been proposed to explain how bacteria achieve pH homeostasis: (i) the buffering capacity of cytoplasm, (ii) low proton permeability, and (iii) the extrusion of protons from the cytoplasm by a membrane-bound proton pump. In a study of six bacterial species, Krulwich et al. (17) concluded that the cytoplasmic buffering capacity plays a very limited role in cellular pH homeostasis. However, it has been argued that low internal pH may induce specific enzymes that may be involved in pH homeostasis (4). To investigate this point, we incubated AD305 at pH 2.5 with and without chloramphenicol (150 μg/ml) and compared the survival rates. The absence of a measurable effect of chloramphenicol on acid tolerance (percent survival) in AD305 for at least 4 h (data not shown) indicated that de novo protein synthesis was not required for acid tolerance. To gain further insight into the acid tolerance, we studied the effect of a weak acid, benzoic acid, on the growth and survival of AD305 and AD324. Unlike strong acids, weak acid freely enters a cell in its undissociated form and dissociates to a proton inside the cytoplasm, depending on ΔpH (difference between outside and cytoplasmic pH). Salmond et al. (21) showed that growth inhibition caused by weak acid, at least in part, was due to the dissociation of these acids inside the cytoplasm. We measured the growth rates of AD305 and AD324 in LB, pH 5.0, in the presence of benzoic acid at 37°C. Although the growth rates of both strains were reduced by 1 mM benzoic acid, the effect was 50% higher in AD324 in an average of two experiments (data not shown). If we assume that the entry and subsequent dissociation of benzoic acid are similar in AD305 and AD324, then it is reasonable to hypothesize that the lower growth inhibition in AD305 was a result of a highly efficient proton pump in this organism. Slower removal of protons from the cytoplasm of AD324 may lead to a higher level of acidification of cytoplasmic components, which in turn would lead to growth inhibition and death. It is also possible that AD324 may have some specific targets (enzymes) that are more sensitive to acidification than those in AD305 (4). Further work is needed to resolve these points.

Bacterial metabolism varies with the growth phase (23). The expression of specific genes has been shown to alter as bacteria enter the stationary growth phase (18). In view of this, we studied growth phase-dependent acid tolerance in EHEC strain AD305. Fresh overnight cultures in LB, pH 7.3, were diluted in the same medium, and incubation was continued at 37°C with gentle shaking. Samples were withdrawn hourly and centrifuged in Eppendorf tubes for 2 min at 15,000 rpm, using a Tomy high-speed microcentrifuge (Peninsula Laboratories, Inc., Belmont, Calif.). The pellets were suspended in equal volumes of LB adjusted to pH levels of 7.3 and 2.5. The pH 7.3 samples were plated immediately on LB agar; the pH 2.5 samples were incubated at 37°C for 2 h before being plated on LB agar. The plates were incubated overnight at 37°C, and CFU were then counted. At each sampling time, the survival percentage was calculated and results were plotted on a semilog graph. Growth phase-dependent acid tolerance experiments with AD305 were done at least three times. Although the individual values varied at times by as much as two- to threefold, the basic shape of the graph remained constant. In a typical experiment (Fig. 2), the acid tolerance of AD305 was

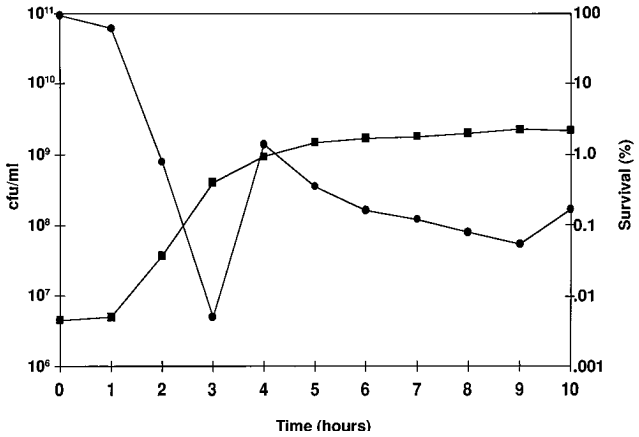

FIG. 2. Growth phase-dependent acid tolerance of EHEC strain AD305. Cells were grown in LB, pH 7.3, at 37°C (■). Survival percentages (●) were calculated after incubation of this culture in LB, pH 2.5, at 37°C for 2 h.

highest at late stationary phase (overnight culture), which decreased several logs when cells were in the mid-exponential phase. A second peak of high acid tolerance, observed at the early stationary phase, was about 100-fold less than that at the late stationary phase. The change in acid tolerance was not due to a change in the pH (7.6) of the overnight culture, which was marginally higher than the pH (7.3) of the culture maintained throughout the experiment (data not shown). If the stationary phase can be compared with the stress conditions caused by either nutrition deprivation or other environmental factors (e.g., temperature, salinity, and pH), it can be assumed that stress caused by these agents will also lead to a higher level of acid tolerance through some global regulatory factor. Similar growth phase-dependent acid tolerance was also observed in *S. flexneri* (12).

EHEC strains have been isolated from a variety of foods and natural environments that provide a wide range of conditions in terms of nutrients, pH, salinity, and temperature. Recently, apple cider and mayonnaise, two acidic foods contaminated with *E. coli* O157:H7, were implicated in outbreaks of diarrhea and hemolytic-uremic syndrome. Several genes that control the adaptive acid tolerance observed in *Salmonella* spp. have been identified (8–10). To determine whether a similar phenomenon exists in EHEC strains, we studied acid tolerance using two different strains of EHEC that were selected on the basis of their acid tolerance: AD305, from a high-tolerance group, and AD314, from a low-tolerance group. The strains were grown to mid-exponential phase (an optical density at 610 nm of 0.6) at 37°C in LB preadjusted to pH 5.0, 6.0, 7.0, and 8.0 with different buffers (Table 2). After incubation, the pH of each culture was recorded and the culture viability in pH 2.5 was assayed as described earlier (Table 2). The pH of the growth medium clearly had a significant effect on subsequent survival in acidic pH: the higher the growth medium pH, the lower the tolerance, and vice versa. However, the effect was not strain specific; both strains showed the effect, but to different extents. The pattern of this adaptive response was similar to that observed in *Salmonella* spp. (10).

TABLE 2. Relationship of growth medium pH and acid tolerance in EHEC strains

Medium pH[a]		Survival (%) of strain[b]:	
Before growth	After growth	AD305	AD314
5.0	4.9	87	51
6.0	6.0	80	0.09
7.0	6.8	47	0.002
8.0	7.5	1.0	0.004

[a] LB was buffered with 100 mM citrate (for pH 5.0), 2-(*N*-morpholino)ethanesulfonic acid (for pH 6.0), 3-(*N*-morpholino)propanesulfonic acid (for pH 7.0), and Trizma (for pH 8.0).

[b] Survival percentages were calculated from viable counts of the cultures after 2 h of incubation at 37°C in LB acidified to pH 2.5 with HCl.

Our studies indicated that many EHEC strains can survive for long periods (5 h) in an acidic pH at 37°C, a situation similar to the human gastric environment. Understanding the differences between highly and slightly acid-tolerant EHEC strains should help to elucidate the mechanism of acid tolerance in this organism. If in vitro acid tolerance studies truly reflect in vivo acid tolerance in gastric juice, then it is clear from our studies that EHEC strains, irrespective of their serotype, will have a low infective dose similar to that of *S. flexneri*. The high level of acid tolerance of some EHEC strains also raises doubts about the efficacy of a proposed acid wash procedure for beef carcasses as a means of reducing O157:H7 contamination (7). The effectiveness of such treatments has been seriously questioned by Brackett et al. (5). Finally, a change in the acid tolerance level as a result of growth phase variation and growth medium pH underscores the importance of the physicochemical environments of contaminated foods in determining the infective dose.

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