

Culturing Enterohemorrhagic *Escherichia coli* in the Presence and Absence of Glucose as a Simple Means of Evaluating the Acid Tolerance of Stationary-Phase Cells

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Prior growth of seven enterohemorrhagic and one nonenterohemorrhagic strains of *Escherichia coli* in tryptic soy broth with (TSB+G) and without (TSB-G) 1% glucose was evaluated for its effect on acid tolerance. The final pHs of 18-h TSB+G and TSB-G cultures were 4.6 to 5.2 and 6.9 to 7.0, respectively. Cells were then transferred to brain heart infusion broth adjusted to pH 2.5 or 3.0 with HCl, incubated at 37°C for up to 7 h, and assayed periodically for viable populations with brain heart infusion and MacConkey agars. All enterohemorrhagic strains were acid resistant (<0.5 log decline after 7 h) when initially cultured in TSB+G, but substantial differences in acid tolerance were observed among strains cultured in TSB-G (log declines ranged from <0.3 to >3.8). The results indicated that prior growth in a medium with and without a fermentable carbohydrate is a convenient way of studying the induction of acid tolerance, that acid inactivation is preceded by a period of acid injury, and that pH-independent and pH-dependent stationary-phase acid tolerance phenotypes may exist among strains of enterohemorrhagic *E. coli*.

One of the physiological characteristics of enterohemorrhagic *Escherichia coli* that is believed to play a key role in food-borne cases of hemorrhagic colitis and its sequelae is the microorganism's acid tolerance. The ability to survive transitory exposure to strong acidic conditions (pH 1.5 to 3.0) and extended exposure to moderate acidic conditions (pH 3.0 to 4.5) is believed to enhance the bacterium's potential for causing disease by two means. First, resistance to strong acid conditions enhances survival of the pathogen during passage through the stomach, thereby decreasing the infectious dose (9, 14). Second, resistance to moderately acidic conditions enhances survival in foods which rely on their low pHs to inactivate pathogens. *E. coli* O157:H7 has been shown to resist inactivation in apple cider (12, 13, 19), mayonnaise (5, 17, 18), and fermented meats (7, 12), three products that have been linked to outbreaks of hemorrhagic colitis and hemolytic-uremic syndrome.

Several physiological systems have been associated with the development of acid resistance in enteric bacteria, with the primary system appearing to differ among species. Separate inducible, pH-dependent systems have been identified for logarithmic- and stationary-phase *Salmonella typhimurium* cells, as has a pH-independent system associated with the stationary phase and the *rpoS* gene (6, 10). Acid resistance in *E. coli* and *Shigella flexneri* was initially reported to be limited to a pH-independent, *rpoS*-associated system (9), but more recently a second pH-dependent system associated with anaerobic growth has been identified (15). While enterohemorrhagic *E. coli* is noted for the ability to survive transitory exposure to acid environments, the factors that influence the induction and extent of acid tolerance in this pathogen have not been established. Arnold and Kasper (1) studied a single strain of *E. coli*

O157:H7 and concluded that acid tolerance (as determined by exposure to synthetic gastric juice and HCl with pHs of 1.5 and 2.0, respectively) was independent of prior exposure to moderate acid conditions. They also observed that starvation increased acid tolerance and that this effect required de novo protein synthesis during the period of starvation. Conversely, Benjamin and Datta (2) reported that the ability of two strains of *E. coli* O157:H7 to resist exposure to pHs 2.5 and 3.0 (HCl) was phase dependent and pH dependent and did not require de novo protein synthesis after transfer to the acidic environment. Examining five strains of *E. coli* O157:H7, Leyer et al. (12) demonstrated that acid habituation induced by culturing the microorganisms in broth with a pH of 5.0 substantially increased the microorganisms' tolerance to subsequent exposure to acidic media and foods. Cheville et al. (4) concluded that the *rpoS* gene is associated with sustained acid tolerance in *E. coli* O157:H7 and that it also influences thermal resistance and salt tolerance and promotes survival in fermented sausage.

The development of effective means for validating food products and processes that rely on acid inactivation of enterohemorrhagic *E. coli* requires that (i) the range of acid tolerance responses (ATRs) and mechanisms among isolates be characterized, (ii) the most resistant strains that are likely to be encountered have been identified, and (iii) means for ensuring that the isolates are in their most resistant state are available. The same information is needed to effectively assess the ability of acid tolerance to induce cross protection in *E. coli* against other stresses such as thermal or irradiation processing. Currently, researchers induce an ATR by transitorily exposing cultures to moderately acidic conditions or growing the cells in acidified medium. The objective of the present study was to determine if the ability of *E. coli* to acidify its environment as a result of glucose fermentation could be used to easily and effectively induce acid tolerance in enterohemorrhagic strains. Seven enterohemorrhagic strains (six O157:H7 and one O111:H⁻) and one nonenterohemorrhagic reference strain were examined, with the strains having been selected to include a range of acid tolerances.

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TABLE 1. *E. coli* strains used in this study

Strain	Serotype	Description and source ^a
B1409	O157:H7	Clinical isolate, CDC
45753-35	O157:H7	Isolate from beef kidney sample, FSIS
30-2C4	O157:H7	Clinical isolate from outbreak associated with dry cured salami, CDC via FSIS
932	O157:H7	Clinical isolate, CDC via M. P. Doyle
Ent-C9490	O157:H7	Clinical isolate from "Jack in the Box" outbreak, CDC
A9124-C1	O157:H7	Clinical isolate, CDC
95JB1	O111:H ⁻	Clinical isolate from outbreak associated with mettwurst, Adelaide Children's Hospital (Adelaide, Australia) via J. Paton
ATCC 25922	Not available	Reference strain, Difco

^a CDC, Centers for Disease Control and Prevention; FSIS, Food Safety & Inspection Service.

MATERIALS AND METHODS

Microorganisms. Six enterohemorrhagic O157:H7 strains, one enterohemorrhagic O111:H⁻ strain, and one nonenterohemorrhagic *E. coli* strain from the Eastern Regional Research Center stock culture collection were used in the study. The identities and sources of the isolates are summarized in Table 1. The six O157:H7 isolates were selected to include a range of acid tolerances on the basis of their ability to survive for extended periods under moderately acidic conditions (pH 4.0 to 4.5). As determined by a preliminary screening (7a), strains 932 and A9124-C1 were moderately acid tolerant, strains B1409 and 45753-35 were strongly acid tolerant, and strains 30-2C4 and Ent-C9490 were extremely acid tolerant. All strains were cultured in tryptic soy broth (TSB) (Difco, Detroit, Mich.) for 24 h at 37°C and then stored at 2°C. These working stock cultures were transferred bimonthly.

A starter culture of each strain was initiated 18 h before the start of an acid challenge by inoculating individual test tubes (16 by 125 mm) containing 10 ml of either TSB without glucose (TSB-G) (Difco) or TSB supplemented with glucose to a concentration of 10 g/liter (1%) (TSB+G). The inocula consisted of 0.1 ml of the working stock cultures. The starter cultures were incubated at 37°C until initiation of the acid tolerance assay.

Assessment of acid tolerance. The acid tolerance of each strain was evaluated on at least two separate occasions for each combination of starter culture medium (TSB+G and TSB-G) and acid challenge pH (pH 2.5 and 3.0). Brain heart infusion broth (BHI) (Difco) was prepared, adjusted to either pH 3.0 or 2.5 with concentrated HCl, and distributed in 10-ml portions to approximately 60 test tubes. The tubes were sealed with plastic caps and sterilized by autoclaving for 20 min at 121°C. The pH of BHI from representative tubes was tested after sterilization to verify that any pH changes resulting from autoclaving were ≤ 0.1 pH unit. All BHI-filled tubes were pre-equilibrated to 37°C.

Sets of six tubes containing pH-adjusted BHI were inoculated with 0.1 ml of starter culture of one of the eight strains. This resulted in an initial level of *E. coli* of approximately 10^7 CFU/ml. One tube was immediately assayed for viable counts (see below), while the rest of the tubes were returned to the 37°C incubator. At designated intervals up to 7 h, one tube per strain was removed and assayed. After all BHI tubes were inoculated, the pHs of the starter cultures were determined with a pH meter.

The contents of the BHI tubes were diluted as needed by transferring 0.1-ml portions to sterile 9.9-ml dilution blanks of 0.1% peptone water. Samples were plated on duplicate BHI agar (BHIA) and MacConkey agar (MA) plates with a Spiral Plater (model 3000; Spiral Biotech, Bethesda, Md.). All plates were incubated at 37°C for 24 h, and then bacteria were enumerated. The BHIA counts were used as a measure of the total number of viable cells, while the MA counts were used to estimate the number of noninjured cells. The level of injured cells (as measured by the loss of resistance to bile salts) was calculated by using the differential between the BHIA and MA counts. Neither the BHIA or MA counts increased with extended incubation. The lower limit of detection for the plating system was a \log_{10} CFU per milliliter of 1.00.

RESULTS

The effect of the glucose concentration (0.0, 2.5, 5.0, 7.5, and 10.0 g/liter) of TSB on the depression of pH by *E. coli* was initially characterized with strain Ent-C9490 (Fig. 1). The presence of glucose resulted in a concentration-dependent depression of pH after autoclaving. The final pH reached by *E. coli* Ent-C9490 was dependent on glucose concentration, with the glucose-free culture remaining close to the initial pH. Maximum depression of pH was observed with glucose levels of

≥ 7.5 g/liter. The pH of the cultures with the lower glucose concentrations tended to increase if the cultures were incubated for longer than 24 h.

On the basis of these results, TSG-G and TSG+G were used to prepare inocula for the rest of the study. The final pHs attained by the starter cultures of the various strains were monitored (data not shown). Differences among strains were noted; the mean final pHs ranged from 4.6 to 5.2 and 6.9 to 7.0 for TSG+G and TSG-G, respectively. Though there were consistent differences among the strains in the final pH reached by the glucose-containing starter cultures, the pH levels were all well within the range that would be expected to produce a strong ATR.

The effects of glucose availability during prior growth of *E. coli* on its subsequent resistance to a 7-h exposure to pHs 2.5 and 3.0 are summarized in Table 2. The strains' responses to the acid challenges were reproducible, with good agreement among the separate trials. The impact of preexposure culture conditions on viable (BHIA) counts varied among the strains. The greatest change in acid tolerance was observed with the nonenterohemorrhagic strain, ATCC 25922. The challenge with pH 2.5 produced approximately a 1.5-log reduction after 7 h with the TSB+G-grown cells, while the challenge with pH 3.0 had no effect. This relative resistance is in contrast to results for the TSB-G-grown cells, for which the population density fell below the lower limit of detection (10 CFU/ml) within 5 h at pH 2.5 and there was an approximate 4.5-log decline with exposure to pH 3.0.

All glucose-grown enterohemorrhagic strains survived the 7-h exposure to BHI at pH 2.5 or 3.0 with little, if any, inactivation. Even at pH 2.5, the most acid-sensitive strains, 932 and A9124-C1, had only a 0.5- to 1.0-log loss of viability. The response of the glucose-free cultures varied, with the strains segregating into three groups. A lack of prior exposure to the moderately acidic conditions associated with glucose fermentation did not affect the acid resistance of strain 30-2C4, Ent-C9490, or 95JB1. At the other extreme, strains 932 and A9124-C1 were strongly affected by preexposure, with almost a 4-log decline in viable counts being observed with the glucose-free cultures at pH 2.5. Strains B1409 and 45753-35 formed an intermediate group as evidenced by a 1- to 2-log decrease in viable counts.

Dual plating on BHIA and MA was used to assess the extent of acid injury as measured by the loss of resistance to bile salts. In general, the BHIA counts at the end of the 7-h challenge

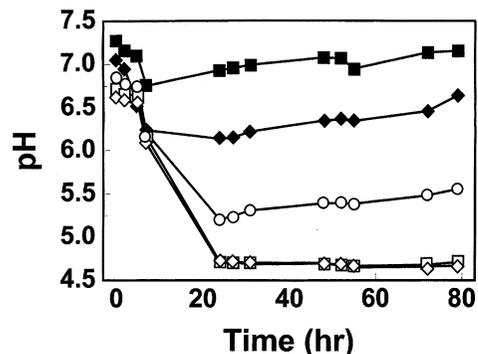


FIG. 1. Effect of glucose concentration of TSB on pH levels attained by *E. coli* Ent-C9490. Values are means of duplicate analysis. Differences among duplicates were always ≤ 0.10 pH unit, with most sample pairs having differences of ≤ 0.02 . Symbols indicate glucose concentrations (in grams per liter) as follows: ■, 0.0; ◆, 2.5; ○, 5.0; □, 7.5; and ◇, 10.0.

TABLE 2. Effect glucose availability on survival of enterohemorrhagic *E. coli* exposed to pHs 2.5 and 3.0

<i>E. coli</i> strain and serotype	Length of exposure (h)	pH 2.5				pH 3.0			
		Count of cells cultured:				Count of cells cultured:			
		With glucose		Without glucose		With glucose		Without glucose	
		BHIA	MA	BHIA	MA	BHIA	MA	BHIA	MA
B1409, O157:H7	0	6.73 ± 0.04	6.57 ± 0.05	6.39 ± 0.05	6.39 ± 0.12	6.64 ± 0.07	6.57 ± 0.15	6.25 ± 0.13	6.47 ± 0.18
	1					6.75 ± 0.07	5.78 ± 0.34	6.04 ± 0.00	5.74 ± 0.18
	2	6.46 ± 0.09	5.66 ± 0.28	5.88 ± 0.20	4.78 ± 0.03				
	3					6.95 ± 0.19	5.71 ± 0.01	5.59 ± 0.37	4.44 ± 0.55
	5	5.69 ± 1.01	4.25 ± 0.74	4.90 ± 0.13	4.33 ± 0.31	6.80 ± 0.14	5.31 ± 0.33	5.08 ± 0.54	3.90 ± 0.69
	7	6.58 ± 0.03	3.94 ± 0.54	4.43 ± 0.12	3.84 ± 0.23	6.82 ± 0.14	5.68 ± 0.41	4.41 ± 0.59	3.39 ± 0.48
45753-35, O157:H7	0	6.73 ± 0.04	5.28 ± 0.37	6.43 ± 0.08	5.90 ± 0.18	6.60 ± 0.03	4.97 ± 0.19	6.23 ± 0.12	5.50 ± 0.09
	1					6.62 ± 0.13	4.74 ± 0.18	6.07 ± 0.01	5.33 ± 0.08
	2	6.48 ± 0.01	4.63 ± 0.24	6.07 ± 0.02	4.98 ± 0.27				
	3					6.54 ± 0.16	4.93 ± 0.00	5.80 ± 0.19	4.84 ± 0.10
	5	5.94 ± 0.47	5.44 ± 0.11	5.60 ± 0.09	4.84 ± 0.30	6.56 ± 0.23	4.56 ± 0.04	5.41 ± 0.32	4.57 ± 0.60
	7	6.25 ± 0.06	5.33 ± 0.21	5.24 ± 0.15	4.58 ± 0.14	6.46 ± 0.32	4.50 ± 0.34	5.02 ± 0.34	4.28 ± 0.20
30-2C4, O157:H7	0	6.66 ± 0.12	6.70 ± 0.13	6.30 ± 0.06	6.16 ± 0.02	6.65 ± 0.01	6.36 ± 0.27	6.04 ± 0.01	6.24 ± 0.18
	1					6.61 ± 0.21	5.97 ± 0.46	6.23 ± 0.06	5.74 ± 0.03
	2	6.66 ± 0.01	5.44 ± 0.25	6.21 ± 0.02	5.28 ± 0.20				
	3					6.73 ± 0.04	6.03 ± 0.34	6.15 ± 0.07	5.30 ± 0.23
	5	6.79 ± 0.08	5.68 ± 0.19	6.15 ± 0.05	5.02 ± 0.14	6.82 ± 0.15	5.85 ± 0.23	6.04 ± 0.08	5.39 ± 0.46
	7	6.65 ± 0.06	5.44 ± 0.20	6.09 ± 0.08	5.04 ± 0.08	6.77 ± 0.17	5.72 ± 0.62	6.01 ± 0.10	4.91 ± 0.11
932, O157:H7	0	6.44 ± 0.42	4.98 ± 0.29	6.39 ± 0.33	5.71 ± 0.49	6.18 ± 0.04	4.88 ± 0.32	5.91 ± 0.08	5.18 ± 0.02
	1					6.21 ± 0.10	4.69 ± 0.13	5.06 ± 0.07	4.47 ± 0.32
	2	6.20 ± 0.49	4.52 ± 0.29	4.77 ± 0.79	4.42 ± 0.46				
	3					5.88 ± 0.19	4.51 ± 0.21	4.83 ± 0.09	3.90 ± 0.26
	5	6.19 ± 0.76	3.82 ± 0.19	3.26 ± 0.30	2.22 ± 0.27	5.53 ± 0.47	4.42 ± 0.16	4.44 ± 0.10	3.52 ± 0.31
	7	6.16 ± 0.73	3.77 ± 0.20	2.59 ± 0.18	1.43 ± 0.58	5.43 ± 0.49	4.44 ± 0.18	4.33 ± 0.11	3.43 ± 0.53
Ent-C9490, O157:H7	0	6.84 ± 0.09	6.83 ± 0.06	6.44 ± 0.02	6.28 ± 0.11	6.60 ± 0.01	6.62 ± 0.01	6.21 ± 0.10	6.36 ± 0.16
	1					6.84 ± 0.23	6.37 ± 0.23	6.11 ± 0.04	6.30 ± 0.06
	2	6.60 ± 0.08	6.18 ± 0.15	6.32 ± 0.01	5.67 ± 0.22				
	3					6.61 ± 0.20	6.57 ± 0.18	6.20 ± 0.06	5.81 ± 0.32
	5	6.73 ± 0.05	6.11 ± 0.14	6.31 ± 0.11	5.27 ± 0.00	6.86 ± 0.24	6.46 ± 0.04	6.13 ± 0.07	5.08 ± 0.18
	7	6.46 ± 0.07	5.90 ± 0.13	6.09 ± 0.11	5.03 ± 0.15	6.85 ± 0.21	6.06 ± 0.50	6.01 ± 0.08	5.04 ± 0.15
A9124, O157:H7	0	6.85 ± 0.07	6.40 ± 0.20	6.51 ± 0.10	6.24 ± 0.07	6.44 ± 0.13	6.21 ± 0.19	6.35 ± 0.01	5.93 ± 0.26
	1					6.60 ± 0.11	6.28 ± 0.18	6.11 ± 0.07	5.03 ± 0.08
	2	6.64 ± 0.18	5.68 ± 0.25	4.72 ± 0.85	3.84 ± 0.47				
	3					6.59 ± 0.16	6.03 ± 0.23	5.25 ± 0.71	4.00 ± 0.38
	5	6.63 ± 0.27	5.05 ± 0.23	3.20 ± 0.03	2.55 ± 0.05	6.71 ± 0.19	5.69 ± 0.34	4.02 ± 0.96	2.54 ± 1.54
	7	6.58 ± 0.19	5.07 ± 0.29	2.76 ± 0.54	1.79 ± 0.08	6.71 ± 0.17	5.53 ± 0.31	2.71 ± 1.41	1.98 ± 0.98
95JB1, O111:H ⁻	0	6.90 ± 0.07	6.55 ± 0.02	6.76 ± 0.26	6.65 ± 0.05	6.76 ± 0.08	6.73 ± 0.06	6.57 ± 0.03	6.46 ± 0.13
	2	6.77 ± 0.27	5.08 ± 0.36	6.66 ± 0.08	6.09 ± 0.05	6.74 ± 0.10	6.38 ± 0.05	6.68 ± 0.10	6.63 ± 0.05
	5	6.76 ± 0.27	5.29 ± 0.10	6.70 ± 0.04	5.37 ± 0.04	7.08 ± 0.10	5.63 ± 0.68	6.76 ± 0.06	6.61 ± 0.03
	7	6.80 ± 0.15	4.51 ± 0.91	6.49 ± 0.02	5.14 ± 0.27	6.89 ± 0.14	5.74 ± 0.58	6.61 ± 0.09	6.27 ± 0.01
ATCC 25922 ^b	0	6.84 ± 0.13	6.31 ± 0.24	6.65 ± 0.05	5.97 ± 0.05	6.74 ± 0.04	6.56 ± 0.04	6.75 ± 0.07	6.64 ± 0.02
	2	6.56 ± 0.18	5.04 ± 0.36	2.90 ± 0.08	1.52 ± 0.05	6.95 ± 0.08	5.33 ± 1.27	4.48 ± 0.03	3.95 ± 0.27
	5	6.19 ± 0.17	4.78 ± 0.67	<1.00	<1.00	6.70 ± 0.12	6.10 ± 0.32	2.69 ± 0.46	1.81 ± 0.81
	7	5.41 ± 0.30	3.93 ± 0.45	<1.00	<1.00	6.71 ± 0.03	6.00 ± 0.35	2.17 ± 0.32	1.50 ± 0.50

^a Organisms were grown in TSB-G or TSB+G and resuspended at 37°C in BHI adjusted to pH 2.5 or 3.0 with HCl. Values are means and standard deviations of at least two independent trials with all assays done in duplicate ($n \geq 4$), and are reported as log CFU per milliliter.

^b Biotype 1 reference strain, serotype unavailable.

period were 0.5 to 1.0 log cycles greater than the corresponding MA counts, indicating that 50 to 90% of the surviving population were sublethally stressed. The differential between BHIA and MA counts was typically greater with the glucose-grown cells, suggesting that preadaptation to moderately acidic conditions may allow injured cells to remain viable longer. An exception was strain 95JB1, one of the three strains with which

TSB+G- and TSB-G-grown cells were equally acid tolerant. In this instance prior growth in the presence of glucose appeared to make the cells more susceptible to injury but did not affect their acid tolerance. Injury generally preceded a loss of viability (e.g., strain A9424-C1 grown in TSB-G and exposed to pH 3.0; however, injury did not always lead to inactivation (e.g., strain 95JB1 grown in TSB+G and TSB-G and exposed

to pH 2.5). Interestingly, strains 45753-35 and 932 often had 1.0- to 1.5-log differentials in BHIA and MA counts both before (data not shown) and immediately after inoculation into acidified BHI. This suggests that >90% of these cells were injured as part of their normal attainment of stationary growth.

DISCUSSION

The use of TSB-G and TSB+G in conjunction with an 18-h incubation proved to be an easy and effective means of evaluating the acid tolerance characteristics of *E. coli*. Supplementation of TSB to $\geq 0.75\%$ glucose ensured that cultures reached and maintained a pH that would foster maximal acid tolerance. Presumably, maximal differentiation of acid-tolerant and non-acid-tolerant states is further ensured by using TSB-G; even the 0.25% glucose present normally in TSB produces a small, if transitory, depression of pH (Fig. 1) that approaches that which induces an ATR in *S. typhimurium* (6). The dose-related decrease in pH associated with autoclaving glucose in TSB has been reported previously (16) but had no appreciable effect on the growth kinetics of the *E. coli* isolates.

Currently, there is some disparity in the literature related to the terminology used to describe the different forms of acid resistance. The terms ATR (6) and acid habituation (8, 12) have been used to describe pH-dependent tolerance, and the term acid resistance has been used to describe pH-independent, phase-dependent tolerance; however, these terms have not been universally accepted. Gordon and Small (9) pragmatically defined acid resistance in *E. coli* and *S. flexneri* as >10% survival when cells are exposed to pH 2.5 for 2 h. When cultured in TSB+G, all eight strains met this criterion. When grown in TSB-G, only the nonenterohemorrhagic strain would have been considered acid sensitive, even though extended exposure produced what appears to be three classes of acid tolerance among enterohemorrhagic strains. This suggests that the criterion of Gordon and Small (9) needs to be modified by extending the exposure time so that degrees of acid resistance can be better differentiated.

The acid challenge conditions used in the present study were selected to model the environmental conditions that the microorganism faces when ingested as part of a moderately buffered meal. Two pHs were examined to better quantify the effect of pH on survival. BHI was selected over simpler systems (e.g., buffer or minimal medium) to more closely resemble the composition of foods associated with the transmission of enterohemorrhagic *E. coli* (i.e., meat products). The relative acid tolerances of the TSB-G-grown cultures of the O157:H7 isolates observed in the present study agree qualitatively with the preliminary categorization of the isolates based on their relative tolerances to moderately acidic conditions. This suggests that preculturing of the isolates in glucose-containing media could be used to ensure that cells have maximal acid tolerance when they are used to assess the effectiveness of food processes that rely on acid inactivation to achieve elimination of the pathogen. We have previously used BHI with 1% glucose as a starter culture medium for *Listeria monocytogenes* in studies that quantified its survival in moderately acidic environments (3).

The results described herein emphasize that media that are even mildly selective (e.g., MA) should not be used to assess the ability of *E. coli* to survive acid environments. Comparison of BHIA and MA counts indicated that within 2 h of exposure to the acid challenge, 90 to 99% of viable cells were injured and would not have been detected if a selective medium had been used. While a loss of viability was generally preceded by injury, the more resistant strains remained viable despite an

extended period of injury. Elucidation of the relationship between injury and loss of viability will require additional research, particularly in relation to differences among strains and the effect of preexposure to an acidic environment.

The response of enterohemorrhagic *E. coli* to the acidic conditions generated as a result of culture in the presence and absence of the fermentable carbohydrate was not uniform among the strains. Strains 932 and A9124-C1 behaved in a manner that resembled that of the nonenterohemorrhagic reference strain. All three were relatively sensitive to the acid challenge when grown in carbohydrate-free medium but acquired substantial tolerance when preadapted. This response appears to resemble the pH-dependent stationary-phase ATR reported for *S. typhimurium* (6, 10) and acid resistance in *E. coli* and *S. flexneri* (15) but is not consistent with the reports that acid resistance in *E. coli* is pH independent (1, 9). Conversely, the lack of enhancement of the acid tolerance in strains 30-2C4, Ent-C9490, and 95JB1 indicates either that acid tolerance in these strains is pH independent (1) or that they are analogous to the constitutively acid-tolerant *S. typhimurium* mutants of parent strains that were initially pH dependent (6, 10). It is interesting that the three strains that had pH-independent acid tolerance were the isolates associated with large outbreaks. This suggests that this characteristic may enhance the isolates' pathogenicity. The intermediate response observed with strains B1409 and 45753-35 suggest that there is a continuum of ATRs. It would be helpful to understanding the role of acid tolerance as a virulence factor if future research determined if these isolates represent three genotypically distinct groups or are simply a spectrum of strains that have differing *rpoS* expression (15) or produce different levels of the same protective cellular components, such as stress proteins (10).

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