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Injury and Recovery of *Escherichia coli* After Sublethal Acidification

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Over 99% of the viable cells of *Escherichia coli* K-12 were injured after a 60-min exposure to 0.3 M sodium acetate buffer at pH 4.2. Injured cells were those able to grow on Trypticase soy agar but unable to grow on violet red bile agar. The extent of both death and injury of acid-treated cells increased with decreasing pH or increasing concentration of acid. Injured cells were able to recover their colony-forming ability on violet red bile agar by incubation in Trypticase soy broth or potassium phosphate buffer before plating on the agar media. Direct neutralization of the injury medium did not allow recovery and, in fact, was lethal to the population. The addition of metabolic inhibitors to the Trypticase soy recovery broth was used to study the repair process. It was not affected by the presence of inhibitors of protein, cell wall, deoxyribonucleic acid, or ribonucleic acid syntheses. The addition of 2,4-dinitrophenol to the recovery medium also did not inhibit recovery. Actinomycin D and *N,N'*-dicyclohexylcarbodiimide were lethal to a proportion of the acidified cells but allowed another fraction of the population to recover. There were no detectable amounts of 260- or 280-nm-absorbing materials leaked during the course of acid injury.

Many food products receive processing or contain agents, natural or added, which impart stress to bacteria present in the product. This stress may not be severe enough to destroy all bacteria present but may, instead, inflict sublethal injury to a proportion of the population. Such injury leaves cells more susceptible to an adverse environment (secondary stress) to which noninjured cells could readily adapt. Secondary stresses are also encountered by sublethally injured cells in the form of selective agents included in enumeration media (2). Injured cells can be defined as those cells which can form colonies on low-stress media but cannot form colonies on stressing media. The injured cells in a population can be quantitatively determined by comparing plate counts on a nonselective medium with plate counts on a selective medium (3). Extended exposure of cells to the primary stress may eventually lead to the death of these microorganisms. However, if the injured cells are removed from stressful conditions, a revitalization of the cells may occur. The restoration of colony-forming ability on selective media is interpreted as a recovery from the initial injury and indicates a repair of those synthetic and metabolic functions damaged during the stress.

Injury to *Escherichia coli* has been observed to occur following heat treatment (5, 11), freezing (8), freeze-drying (13), and irradiation (1, 6). The investigation of acid injury upon *E. coli* has been limited to showing the loss of colony-forming ability on violet red bile agar (VRBA; 10). Acid injury has also been observed in *Staphylococcus aureus* without a further investigation of recovery or mechanism (7).

Since exposure to acidic conditions is commonly encountered by bacteria in foods, such bacteria are likely to be acid injured. This study was designed to estimate the extent of the injury produced by acidic environments, to determine the conditions for resuscitation of acid injury, and to attempt to characterize the mode of action of acid injury.

MATERIALS AND METHODS

Test organism. Actively growing cells of *E. coli* K-12 were inoculated into fresh Trypticase soy broth (TSB), grown at 32°C to an absorbance of 1.0 (approximately 10^9 cells per ml), and harvested by centrifugation at 4,000 × *g* for 5 min at 0 to 2°C. The supernatant was decanted, and the cells were suspended in a volume of 0.1% peptone water equal to that of the growth medium.

Injury and recovery media. Sodium acetate buffers were prepared with distilled water to the appropriate concentrations and pH levels and sterilized by filtration through a 0.45-μm membrane filter unit. Volumes (99 ml) of the solutions were aseptically transferred to 250-ml Erlenmeyer flasks and equilibrated at 32°C before use in injury studies. Potassium phosphate buffers, 0.1% peptone water, TSB, and TSB plus various metabolic inhibitors were used in recovery

studies. Potassium phosphate buffers were sterilized by filtration, whereas peptone water and TSB were sterilized by autoclaving at 121°C for 15 min. Inhibitors were prepared as stock solutions, filter sterilized, and added to previously sterilized TSB.

Enumeration media. Both injury and recovery were monitored by plating on VRBA as the selective medium and Trypticase soy agar (TSA) as the nonselective medium. All dilutions for plating were prepared in 0.1% peptone water.

Injury procedure. A 1-ml portion of the resuspended culture was added to the injury medium and agitated vigorously for 15 s, and a zero-time sample was withdrawn and plated. The injury vessel was incubated at 32°C on a rotary shaker (125 rpm). Successive 1-ml portions of the acid-treated cell suspension were removed by pipette at 15-min intervals and transferred to 9 or 99 ml of the diluent, and 0.1-ml samples of the resultant dilutions were surface plated immediately, in triplicate, on TSA and VRBA. The inoculated plates were incubated at 32°C for 24 h, and the colony counts were determined. The injured population was measured by the difference in counts between the nonselective and selective media. The percent injury and the *D* values were determined by using the results obtained from the zero-time and 60-min samplings.

Recovery procedure. After 60 min of exposure to the injury medium, a 1-ml portion of the cell suspension was withdrawn and transferred to the recovery medium. The medium was agitated vigorously for 15 s, and a zero-time sample was withdrawn and plated on both TSA and VRBA. When recovery occurred so rapidly as to obscure the actual extent of injury at the zero-time plating, a true zero-time sample was withdrawn directly from the injury vessel, diluted, and plated before addition to the recovery medium. The difference between the zero-time and true zero-time samples was only that the true zero-time sample had not been exposed to the recovery medium. The recovery vessel was incubated at 32°C on a rotary shaker (125 rpm). Successive 1-ml portions were removed by pipette at 20-min intervals and transferred to 9 or 99 ml of diluent, and 0.1-ml samples of the resultant dilutions were surface plated immediately in triplicate. The inoculated plates were incubated at 32°C for 24 h, and the colony counts were determined. Recovery was expressed by an increase in counts on VRBA without an increase on TSA. A simultaneous increase in counts on both the selective and nonselective media expressed growth and not recovery.

Chemicals. The following compounds were used in this study: chloramphenicol, D-cycloserine, hydroxyurea, and 5-fluorouracil from Sigma Chemical Co., St. Louis, Mo.; *N,N'*-dicyclohexylcarbodiimide (DCCD) and 2,4-dinitrophenol from Eastman Kodak Co., Rochester, N.Y.; and actinomycin D from Calbiochem, Los Angeles, Calif. DCCD was added to sterilized TSB as a small volume of stock solution in absolute ethanol. The final ethanol concentration in the recovery medium was 0.2%.

RESULTS AND DISCUSSION

Injury and death due to acidification.

Cells of *E. coli* which were not exposed to acid were able to form colonies on TSA and VRBA with equal ease. However, when these cells were held for 60 min in a 0.3 M sodium acetate buffer at pH 4.2, 99.2% of the viable population as measured on TSA was unable to form colonies on the selective medium VRBA (Fig. 1). *D* values and percent injury to the population from 60-min exposures to varying concentrations and pH levels of sodium acetate buffer are given in Table 1. Exposure either to increasing concentrations of acetate and, hence, undissociated acid at constant pH or to decreasing pH at a constant acetate concentration (which also increases the concentration of undissociated acid) caused an increase in both death and injury.

Recovery in complex media. When 1 ml of

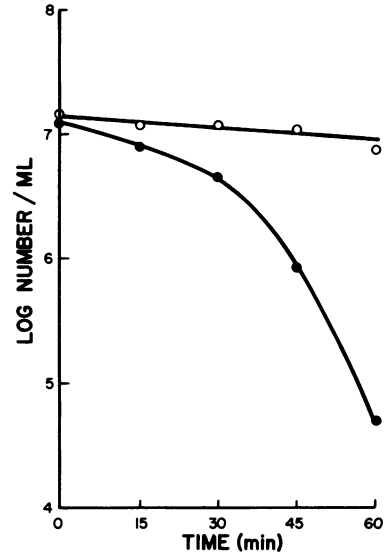

FIG. 1. *Effect on E. coli K-12 of exposure to 0.3 M sodium acetate buffer at pH 4.2. Samples were surface plated on TSA* (○) *and VRBA* (●).

TABLE 1. *Effect of exposure of E. coli to sodium acetate buffers for 60 min*

Concn (M)	pH	*D* value (min)		% Of cells injured
		On TSA	On VRBA	
0.1	4.6	>1,000	186	52.4
0.2	4.6	>1,000	182	65.4
0.3	4.6	>1,000	154	71.0
0.4	4.6	158	27	99.1
1.0	4.6	<19	<0.1	
0.3	4.8	>1,000	380	30.3
0.3	4.6	>1,000	154	71.0
0.3	4.4	>1,000	79	86.0
0.3	4.2	200	26	99.2

cells injured for 60 min in 0.3 M sodium acetate buffer (pH 4.2) was incubated in 99 ml of TSB, recovery occurred (Fig. 2). Complete repair, as indicated by equal colony counts on TSA and VRBA, was observed after 1 to 2 h of incubation and was followed by growth. The addition of 0.3% yeast extract to the recovery medium did not increase the degree of recovery.

Recovery in inorganic buffers. The effect of incubation in various phosphate buffers on recovery from acid injury is given in Table 2. Surprisingly, almost complete recovery (95%) was found after 120 min of incubation in potassium phosphate buffer at a final pH of 8.0. Freeze-injured *E. coli* cells have also been shown to recover in potassium phosphate buffer (9). A sample taken directly from the injury medium after 60 min of exposure to 0.3 M sodium acetate buffer at pH 4.2 showed an injury of 99.95%. A further sample was withdrawn from the injury medium, added to the potassium phosphate buffer recovery medium, and swirled for 15 to 30 s to insure dispersion; a recovery sample was then withdrawn, diluted, and plated. The results of this plating showed an injury of only 95% (5% recovery). Even this slight recovery represented a 100-fold increase in counts on the VRBA medium. After 20 min of incubation in the buffer, 79% of the population was able to form colonies on VRBA, and after 2 h of incubation nearly complete recovery was observed. Recovery at a pH of 7.6 was essentially the same as that at a pH of 8.0 for the first hour or for the major portion of the total recovery. Recovery at a pH of 7.0 and at an increased potassium phosphate concentration was substantially reduced. The potassium could not be effectively replaced by sodium in the phosphate buffer recovery medium. Also, effective recovery was not observed by incubation of injured *E. coli* cells in 0.08 M potassium hydroxide at pH 7.2 or by direct neutralization of the injury medium to an end point of pH 7.0 with sodium hydroxide and further incubation.

Effect of metabolic inhibitors on recovery. A 1-ml inoculum of *E. coli* cells injured by 60 min of exposure to 0.3 M sodium acetate buffer, pH 4.2, was withdrawn and added to 99 ml of TSB containing specific metabolic inhibitors. Results of the effect of these inhibitors on recovery are presented in Table 3. Recovery was

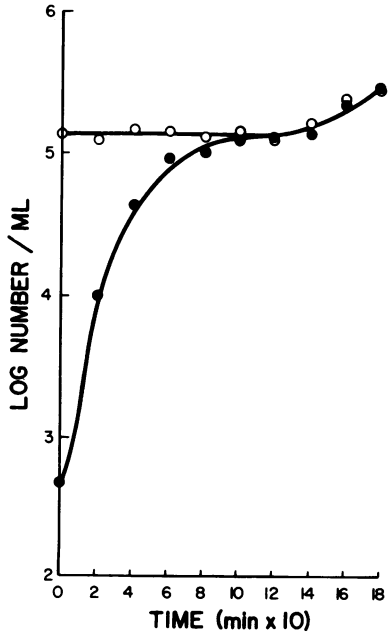

FIG. 2. *Recovery of acid-injured E. coli K-12 in TSB. A 1-ml amount of cells injured by exposure to 0.3 M sodium acetate buffer at pH 4.2 for 60 min was incubated in 99 ml of TSB and surface plated on TSA* (○) *and VRBA* (●).

TABLE 2. *Effect of incubation in potassium phosphate buffer and sodium phosphate buffer on recovery of E. coli after 60 min of exposure to 0.3 M sodium acetate buffer at pH 4.2*

Medium	Concn (M)	Final pH	% Recovery after incubation for:			
			0 min	20 min	60 min	120 min
Potassium phosphate buffer	0.04	8.0	5.0	79.2	86.7	95.0
Potassium phosphate buffer	0.04	7.6	0.31	79.0	87.9	76.5
Potassium phosphate buffer	0.06	7.0	0.85	40.8	55.1	26.6
Sodium phosphate buffer	0.04	8.0	7.3	27.8	24.7	24.9
Sodium phosphate buffer	0.3	7.0	0	0	0	0

TABLE 3. *Effect of inhibitors on recovery of E. coli after 60 min of exposure to 0.3 M sodium acetate buffer at pH 4.2*

Inhibitor	% Recovery after incubation for:			
	0 min	20 min	60 min	120 min
Control[a]	0.35	8.2	68	100
Chloramphenicol	0.13	39	55	74
D-Cycloserine	0.16	28	61	100
Hydroxyurea	<0.01	3.9	31	73
5-Fluorouracil	<0.01	2.6	25	60
2,4-Dinitrophenol	0.26	10	44	72
Streptomycin	8.2	7.3	2.6	3.9
DCCD	0.01	39	90	100
Actinomycin D	0.25	29	36	41

[a] A 1-ml amount of injury inoculum added to 99 ml of TSB.

not prevented by inhibitors of the synthesis of proteins (chloramphenicol at 20 μg/ml), mucopeptides or cell walls (D-cycloserine at 750 μg/ml), deoxyribonucleic acid (hydroxyurea at 500 μg/ml, or ribonucleic acid (5-fluorouracil at 75 μg/ml and actinomycin D at 5 μg/ml). Recovery was also able to proceed when the classical uncoupling agent 2,4-dinitrophenol (100 μg/ml) was present in the recovery medium. Freeze-injured cells of *E. coli* also recovered in the presence of inhibitors of protein, ribonucleic acid, and mucopeptide synthesis (9), but appeared to be more sensitive to 2,4-dinitrophenol uncoupling than did acid-injured cells.

DCCD inhibits a number of energy-dependent transport processes, including the accumulation of potassium by exchange for sodium and hydrogen ions (4). When added to the TSB recovery medium, DCCD (2 mg/ml) produced the recovery curve shown in Fig. 3. A 99.5% death, as determined by reduction in colony counts on the nonselective agar, was observed over the first 40-min sampling period, which leveled off with no apparent decrease over the next 80 min. During the first 40-min sampling period, however, an increase in colony counts on the selective agar was observed. Although the results shown in Fig. 3 translate into 100% recovery as listed in Table 3, it is clear that DCCD had an effect on the recovery system as a whole. Actinomycin D showed similar curves where there was a drop in TSA counts and a rise in VRBA counts. These curves were repeatable.

Streptomycin (10 μg/ml) was the only inhibitor added to the recovery medium that clearly inhibited recovery of acid-injured cells. Which of its many inhibitory mechanisms was responsible was not confirmed. Streptomycin is reported to affect membrane function, including K^+ efflux from the cell, to bind to the ribosomes, and to modify every measurable phase of protein synthesis (12). The most likely function in acid-injured cells is that of membrane modification.

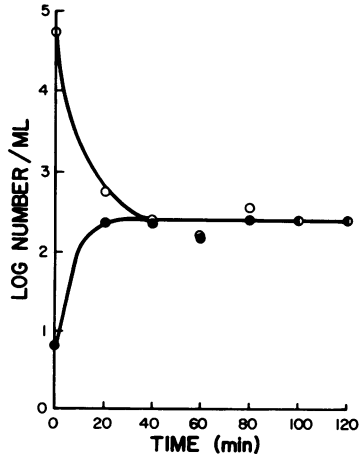

FIG. 3. *Recovery of acid-injured E. coli K-12 in TSB plus DCCD (2 mg/ml). A 1-ml amount of cells injured by exposure to 0.3 M sodium acetate buffer at pH 4.2 for 60 min was incubated in 99 ml of TSB plus DCCD and surface plated on TSA* (○) *and VRBA* (●).

Leakage of cellular constituents. After a 60-min exposure of the test organism to 0.3 M sodium acetate buffer at pH 4.2, there were no detectable amounts of 260- or 280-nm-absorbing materials present in the buffer. Absorbance was measured with a Gilford model 222 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

In conclusion, acid injury has thus been observed to be very different than other types of injury previously studied. The exceptionally rapid recovery from acidification is in great contrast to the time needed for the recovery of freeze-dried *E. coli* (13) or thermally injured *Salmonella typhimurium* (14). The universally observed leakage of cellular constituents following injury (15) was not seen after acidification. Damage to ribonucleic acid has been a constant factor in injury, yet when this synthesis was inhibited, recovery proceeded normally. Finally, the ability of acid-injured cells to recover in media nonsupportive to growth and totally lacking an energy source describes an injury system unlike others. Acid injury appears to be the result of some easily reversible equilibrium shift, which is, however, more complex than simple neutralization of the acid.

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