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Sublethal Stress in *Escherichia coli*: a Function of Salinity†

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Sublethal stress in *Escherichia coli* was detected in various test media after exposure (in vitro) to seawater of various salinities. Stress was measured with an electrochemical detection technique and a β-galactosidase assay. Test media included EC medium, medium A-1, and tryptic soy broth modified to contain lactose for β-galactosidase assay experiments. Stress was defined as the difference between a predicted electrochemical response time calculated for unstarved cells from a standard curve and the observed electrochemical response time for cells starved in seawater. The higher the salinity, the greater the stress for all test media examined. Stress was most pronounced in EC and was attributed primarily to initial die-off of starved cells exposed to the test medium at the elevated temperature of 44.5°C. Lag time and growth rates in test media were not significantly affected by salinity. β-Galactosidase specific activity, assayed in starved cells after transfer to an induction medium at 44.5°C for 150 min, was inversely related to the salinity of the starved cell suspension. The consequences of these observations with respect to coliform enumeration methods are discussed.

Coliform bacteria appear to exhibit rapid die-off when exposed to natural aquatic environments. Attempts to relate die-off to various physicochemical or biological factors have typically involved determinations with an all-or-none enumeration method such as viable count (4, 7, 8, 14, 19). Recently, investigators have become aware of the existence of sublethal stress in coliforms exposed to unfavorable conditions such those as found in aquatic environments (2, 6, 9, 10, 18) or in frozen foods (17). Olson (13) suggested that injured coliforms may not be detected by the standard most probable number procedure. Thus, investigators may have overestimated coliform die-off by enumeration procedures which result in death of sublethally stressed cells.

Two techniques for rapid enumeration of coliforms from aquatic environments have recently been proposed. Wilkins and co-workers (22-24) developed an electrochemical detection method for measuring the time required for a bacterial inoculum to produce a potential increase. Electrochemical detection time (EDT) was inversely related to initial cell density. Similarly, Warren and colleagues (20) developed a β-galactosidase assay method for coliform enumeration based on colorimetric determination of *o*-nitrophenol. Both groups of investigators noted inconsistent results with coliforms from unfavorable environments, suggesting the presence of sublethally stressed cells. Because these techniques measure graded responses, both appeared applicable to the detection of stressed cells.

This paper describes our studies with the EDT and β-galactosidase methods for identifying sublethal stress in *Escherichia coli* after exposure to seawater of selected salinities.

MATERIALS AND METHODS

Organisms. Fecal coliforms were freshly isolated from human feces by enrichment in lactose broth at 35°C for 24 h, followed by inoculation into EC broth at 44.5°C for 24 h. Isolates producing gas in EC were identified by the API 20E (Analytab Products, Inc., Plainview, N.Y.). Two strains, *E. coli* A and B, were maintained on tryptic soy agar (TSA) at 4°C and subcultured on TSA three times or less during the course of experimentation.

Preparation of seawater inocula. Before seawater survival experiments isolates were passed twice in M9 minimal medium (12) (pH 7.0) which consisted of 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, 1 g of NH_4Cl, 0.13 g of $MgSO_4 \cdot 7H_2O$, and 5 ml of glycerol in 1 liter of distilled water. The latter two components were filter sterilized and added to the basal medium after autoclaving. Glycerol was used as the energy source to minimize endogenous metabolism during starvation. After incubation for 18 h at 35°C, bacteria

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were harvested by centrifugation (1,000 × *g*) at room temperature for 10 min and washed three times in phosphate-buffered water (pH 7.2). Cells were resuspended to an optical density at 540 nm (OD_{540}) of 0.2 to 0.3 in seawater (pH 7.4 to 7.6) of selected salinities.

Seawater experiments. A 10-fold dilution of the final washed cell suspension was made in 90 ml of seawater previously equilibrated to the test temperature in 125-ml sterile screw-capped flasks. Seawater at selected salinities was prepared by diluting aged, filtered ocean water (35‰) with reverse osmosis–glass-distilled water and sterilized by autoclaving. Seawater from the mid-Atlantic continental shelf was the sole source for all procedures. Samples were removed after various time intervals for enumeration by spread plating on TSA, determination of EDT, and induction of β-galactosidase. Media used for both EDT and β-galactosidase studies included EC, medium A-1 (1), and Trypticase soy broth (TSB). For enzyme studies, TSB was modified to contain lactose instead of glucose. All tests were run at 44.5°C. Colony counts were made after ca. 24 h of incubation at 35°C. Total exposure time to seawater before zero time sampling ranged from 2 to 5 min, whereas exposure time to media at 44.5°C before dilution for plate counts was less than 30 s.

Determination of EDT. Instrumentation for determining EDT consisted of a test tube (25 by 100 mm) containing two platinum electrodes and 18 ml of medium to which 2 ml of *E. coli*-seeded seawater was added. Tubes were fitted with a no. 4 rubber stopper containing electrodes of grade A platinum alloy wire, 24 gauge (0.508 mm) (Engelhard Industry, Carteret, N.J.). Electrodes were inserted into slits made in the stopper, and the stopper was bound with wire or epoxy or both to prevent electrode slippage. Electrodes were designed such that the ratio of their lengths below the surface of the medium was ca. 1:4. Sterilization was by flaming over an alcohol lamp. The longer electrode was connected to the negative terminal and the shorter one was connected to the positive terminal of a strip chart recorder (model 194, Honeywell Industrial Division, Fort Washington, Pa.; model SR-204, Heath Co., Benton Harbor, Mich.; or model 320, Sanborn Co., Waltham, Mass.). EDT was measured as the time elapsed between challenge and the maximum change in potential.

β-Galactosidase assay. Cells exposed to seawater for various time intervals were diluted 10-fold into EC, medium A-1, and TSB, each containing 0.5% lactose. After 150 min of induction, two 10-ml portions were filtered through 25-mm Nuclepore filters (0.4 μm) with 10-ml hypodermic syringes equipped with Swinny adapters. One portion was washed once with phosphate-buffered water containing magnesium sulfate (0.13 g/liter) and assayed by the *o*-nitrophenyl-β-D-galactopyranoside (ONPG) method (15). The sample was resuspended in 1 ml of a tris(hydroxymethyl)aminomethane (Tris)-magnesium sulfate buffer (Tris, 0.01 M; $MgSO_4 \cdot 7H_2O$, 0.01 M; NaCl, 0.05 M; adjusted to pH 7.4). After addition of 1 drop of toluene, the sample was blended in a vortex mixer and incubated at 35°C for 30 min. A 0.2-ml amount of ONPG (0.13 M in Tris-magnesium buffer, pH 7.4) was added, and the sample was incubated at 28°C until a yellow color developed. The reaction was stopped by addition of 0.5 ml of 1 M Na_2CO_3. OD was measured at both 550 and 420 nm with a Bausch and Lomb Spectronic 70. One unit of enzyme was defined as the amount of enzyme that released 1 nmol of *o*-nitrophenol per min at 28°C, pH 7.4. Enzyme units were calculated from the equation enzyme units = $[OD_{420} - (1.65 \cdot OD_{550})]/[(\text{time of incubation, minutes})(0.0075)]$.

The second 10-ml portion was assayed for protein by the method of Lowry et al. (11). The sample was washed twice with phosphate-magnesium sulfate buffer and resuspended in 1 ml of 1 N NaOH before assay. A standard curve for protein was developed with serum albumin (1 mg/ml in water). Dilutions of serum albumin were made in 0.1 N NaOH. Enzyme specific activity was defined as enzyme units per microgram of protein.

Standard curves and data analysis. The relationship of EDT to inoculum density for unstarved cells was determined with EC, A-1, and TSB media. Tenfold dilutions of an *E. coli* suspension, prepared as described for seawater experiments, were made in 25‰ seawater at 20°C. Exposure to seawater never exceeded 5 min. After samples for initial viable counts were removed, dilutions were inoculated into each test medium and incubated at 44.5°C for determination of EDT. A minimum of 18 EDT measurements were performed to construct a standard curve for each test medium. Data were analyzed by a linear least-squares regression technique with a 95% confidence interval (16).

Standard curve data were used as follows. During exposure of cells to seawater (starvation), samples were removed at timed intervals for determination of viable counts (TSA) and EDT in test media. Viable counts were used to derive a predicted EDT from the appropriate standard curve. Differences obtained by subtraction of the predicted from the observed values were plotted for each test medium as a function of exposure time in seawater.

RESULTS

Effect on viability. Survival studies of *E. coli* in seawater of selected salinities (Table 1) indicated that decreasing salinity was accompanied by increasing survival. At salinities of 15 and 30‰, maximum loss in viability occurred during the first 2 days of exposure, whereas at 10‰ the death rate was appreciably slower.

Effect on EDT. Standard curve data (Table 2) were used to evaluate the responses of starved cell populations upon transfer to media at 44.5°C. Stress, defined as observed minus predicted EDT, was a function of both salinity and test medium (Fig. 1). Generally, maximum stress developed early during the starvation period and subsequently decreased or remained constant after extended exposure. Maximum delay in detection time occurred in EC medium after exposure to 30‰ seawater. Medium A-1 was su-

perior to EC in enumerating stressed *E. coli* and was as effective as TSB. Differences in enumeration efficiency for the three media were most obvious early in the starvation period and diminished with extended starvation.

Effect on growth rate. Growth rates of *E. coli* in different test media were measured with inocula before and after starvation for various time intervals in 10 and 30‰ seawater. As noted above, some loss of viability occurred during exposure to seawater (Table 1). After inoculation into test media, there was an immediate die-off related to duration of exposure to seawater, salinity, and test medium (Table 3). Die-off was most pronounced when cells exposed to 30‰ seawater were inoculated into EC medium. Inoculum reduction was less extensive in medium A-1 and negligible in TSB. Die-off was evident after only 30 s of exposure to the test medium. Cell densities at zero time shown in Fig. 2 reflected both loss of viability in seawater and immediate die-off upon exposure to the test medium at 44.5°C. Thereafter, no obvious differences in lag times or growth rates for any of the inocula were observed.

Effect on β-galactosidase activity after induction in TSB-lactose, medium A-1, and EC. β-Galactosidase specific activity declined with increasing starvation time and salinity (Fig. 3). In cells exposed to 2‰ seawater, enzyme activity decreased slightly in those cells induced in TSB-lactose and not in cells induced in EC or medium A-1. Enzyme activities declined approximately 50 to 80% after 7 days in 10‰ seawater regardless of induction medium. Cells starved in 30‰ seawater evidenced a precipitous decline in specific activity in all three media.

TABLE 1. *Percent survival of E. coli A in seawater adjusted to selected salinities*

Salinity (‰)	Exposure (days)	% Survival[a]
10	2	100.6
	5	87.6
	8	53.5
15	2	27.9
	5	11.7
	8	7.1
25	2	8.6
	5	5.1
	8	4.3
30	2	1.7
	5	0.7
	8	2.0

[a] Based on zero time densities of *E. coli* in seawater (ca. 10^7 cells per ml). Each data point represents the mean of two experiments.

DISCUSSION

Considerable controversy remains concerning causes for the die-off of *E. coli* in aquatic environments. Die-off has most often been measured by viable count techniques employing standard

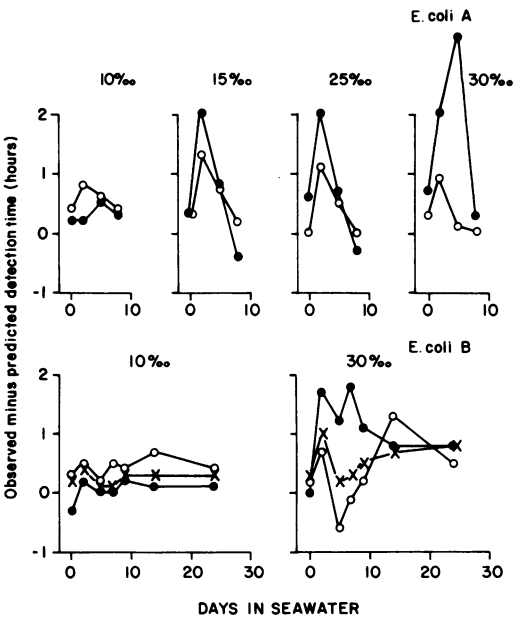

FIG. 1. *Incidence of stress in E. coli during exposure to seawater as measured by the electrochemical detection method. Stress was defined as the difference between observed EDT in test media and predicted EDT based on standard curves. The predicted EDT was derived from a linear regression line based on the viable count in the seawater sample.*

TABLE 2. *Summary of standard curve data in various media at 44.5°C*

Test organism	Test medium	No. of samples	Slope	Detection time intercept (b)	Correlation coefficient	95% Confidence limits
E. coli A	EC	20	−1.23	10.94	−0.99	±0.50
	TSB	22	−1.04	9.85	−0.97	±0.56
E. coli B	EC	18	−1.05	10.26	−1.00	±0.11
	A-1	18	−0.80	8.11	−1.00	±0.13
	TSB	18	−0.92	8.99	−1.00	±0.22

TABLE 3. *Die-off of E. coli B in test media at 44.5°C after exposure to seawater*

Salinity (‰)	Exposure (days)	% Die-off[a] in:		
		EC	A-1	TSB
10	0	0.0	9.1	0.0
	2	21.4	28.6	14.3
	5	0.0	0.0	0.0
	7	0.0	0.0	0.0
	9	5.6	11.1	0.0
	14	13.3	13.3	0.0
	24	7.7	7.7	0.0
30	0	62.3	29.2	0.0
	2	98.8	90.7	11.1
	5	98.1	56.6	11.3
	7	98.9	61.7	5.6
	9	95.9	39.4	12.7
	14	66.9	0.0	0.0
	24	77.1	35.3	23.5

[a] The occurrence of die-off in a given test medium was detected by comparison of viable counts (on TSA) of *E. coli* taken from seawater for immediate enumeration and those enumerated after a 30-s passage through a given test medium at 44.5°C. Percent die-off = [1 − (viable count from test medium/viable count from seawater)] × 100.

media. Recently, it has been noted that the enumeration process itself may be responsible for this apparent die-off in natural waters. Olson (13) noted that more than half of the water samples from California beaches produced false negatives in the standard fecal coliform most probable number test, i.e., growth but no gas in the presumptive portion of the test followed by growth and gas production in both the confirmed and elevated temperature portions of the test. These results suggested that a proportion of the fecal coliforms isolated may have been sublethally damaged and, therefore, would be undetected by usual enumeration procedures.

Bissonnette et al. (2) suggested that coliforms introduced into an aquatic environment exhibit the following responses: death, survival with enumeration possible by standard procedures, and sublethal stress followed by death in standard enumeration media. Stressed cells were identified by their ability to grow on a nonselective, but not on a selective medium. Similarly, Klein and Wu (10) detected the presence of sublethal stress in bacteria starved in freshwater by subjecting the cells to a transient warming stress before plating on nutrient agar.

With the recognition of sublethal stress, efforts have been directed toward development of enumeration procedures which retain selectivity for fecal coliforms but allow recovery of stressed cells. Stuart et al. (18) developed a bilayer plate containing both a selective and a nonselective medium for recovery of fecal coliforms stressed by the aquatic environment. Speck et al. (17) developed a similar bilayer plating procedure for enumerating injured coliforms in frozen foods.

Many workers have attempted inconclusively to evaluate the influence of salinity on *E. coli* die-off in marine environments (5, 7, 14, 19). Rather than employing an all-or-none response to determine the effect of salinity, we chose instead to measure graded responses, i.e., development of an increase in potential, enzyme specific activity, and growth rate in selective and nonselective media. It has been our experience that source, storage conditions, and cultural history of the organisms greatly influenced their response to experimental salinity regimes (unpublished data). Sublethal stress was most apparent in freshly isolated *E. coli* as opposed to isolates stored for long periods of time either on slants at 5°C or in 40% glycerol at −5°C. *E. coli* pregrown in a rich medium (TSB) and then exposed to seawater exhibited a faster, more extensive die-off when tested in a selective medium (EC) than did cells pregrown in a minimal medium. Graded responses were measured with two standard selective media, EC and medium A-1 at 44.5°C, to maximize the expression of stress. TSB served as a nonselective test medium to minimize the expression of stress.

Results with the EDT technique confirm the observation of Wilkins and Boykin (23) that

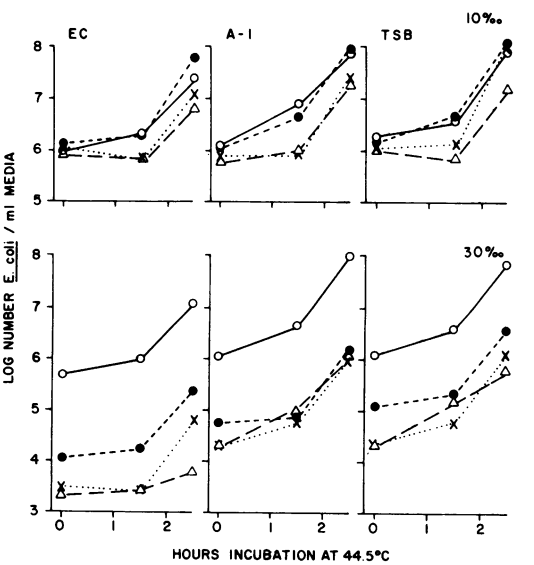

FIG. 2. *Growth of E. coli B in test media at 44.5°C before and after starvation in 10 and 30‰ seawater. Symbols: ○, 0 day; ●, 2 days; ×, 5 days; △, 7 days of starvation.*

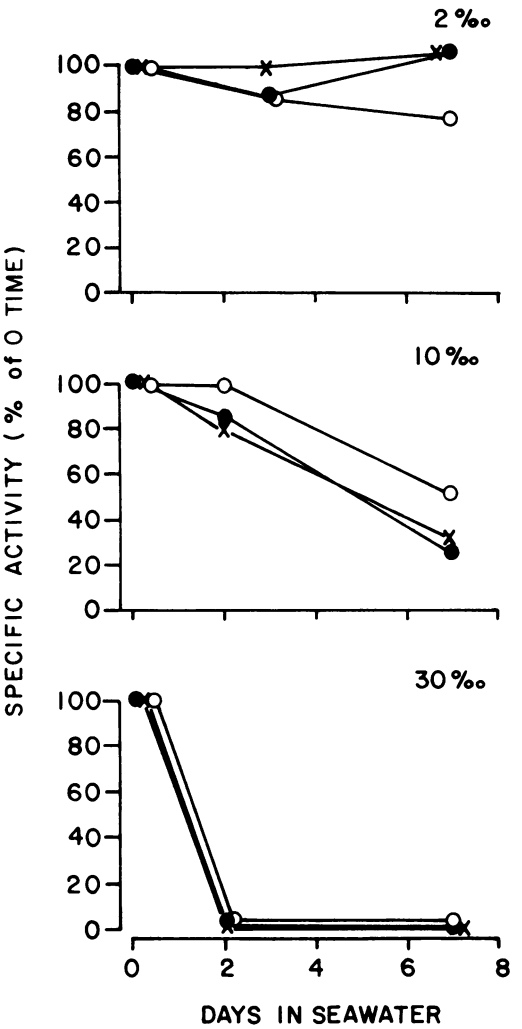

FIG. 3. *Effect of starvation at selected salinities on enzyme specific activity expressed as percentage of zero time activity. Enzyme was induced in various media at 44.5°C. Symbols: ●, EC; ○, TSB; ×, A-1.*

coliforms from estuarine waters exhibit delayed detection times relative to those from freshwater. Cells stressed by in vitro exposure to high salinity displayed EDT responses later than predicted from standard curves. Of the two selective media, stress was considerably more pronounced in EC than in medium A-1. Based on our data, we suggest that the EDT technique would be suitable for coliform enumeration as suggested by Wilkins and Boykin (23) provided that: (i) standard curves are developed for the particular conditions in the environment being monitored; (ii) fecal coliforms isolated from natural waters are used in establishing these standard curves; (iii) medium A-1 or a similar benign yet selective medium is utilized for determination of EDT. The likelihood of underestimating coliform densities would increase if samples were taken from high-stress areas, for example, downstream of a sewage treatment plant or industrial outfall. A possible modification of the EDT technique to improve detection of stressed coliforms, for example, would utilize preincubation at a temperature lower than 44.5°C or in a medium conducive to repair of damaged cells before exposure to selective conditions.

Additional work is necessary to compare the efficiency of the EDT technique to standard most probable number or filtration tests for enumeration of stressed fecal coliforms. The application of the EDT techniques to enumeration of other organisms of public health concern, e.g., pseudomonads, vibrios, fecal streptococci, and staphylococci should also be investigated.

Warren et al. (20), applying the β-galactosidase assay technique to the rapid enumeration of fecal coliforms in natural waters, noted unexpected results when samples were taken from a high-stress area, i.e., downstream of a sewage treatment plant. Our experiment to quantify the extent of sublethal stress in fecal coliforms exposed to seawater measured β-galactosidase specific activity. In contrast, Warren et al. (20) determined the time necessary to reach half-maximum absorbance in ONPG medium. The sharp decrease in specific activity observed in all test media after exposure to seawater may be accounted for by die-off of the coliforms in the test media, catabolite repression, reduction in lactose permease activity, repression of the lactose operon, or a combination of the above. Our data suggested that die-off alone did not account for the decreases in enzyme specific activities since there was minimal die-off in TSB, yet the enzyme activity fell off as sharply as in EC or medium A-1. Similarly, catabolite repression would be expected to reduce enzyme specific activity in rich media where the growth rate or metabolic activity has been depressed; yet there were no clear differences in lag times or growth rates of cells exposed to seawater for varying periods of time (Fig. 2). No attempt was made to measure a decrease in lactose permease or in transcriptional activity.

By using either the EDT or β-galactosidase assay technique, it is clear that sublethal stress can develop as a result of in vitro exposure to autoclaved seawater of selected salinities. These techniques may allow for identification of other potential stress factors such as temperature, nutrient concentration, and active halogen in the aquatic environment. Such information may be

applied to modification of current enumeration techniques allowing for improved accuracy in the estimation of coliform numbers in natural environments.

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