Sensitivity to Bile Salts of Shigella flexneri Sublethally Heat Stressed in Buffer or Broth

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Batch cultures of Shigella flexneri M4243 were grown at 37°C in broth to early stationary phase, washed, and heated at 50°C in 0.1 M phosphate buffer (pH 7.0). Cells were surface plated on a tryptic phytone glucose agar (TPGA), TPGA with 0.15 or 0.85% bile salts no. 3 (TPGA-BS 0.15 or TPGA-BS 0.85), or TPGA with 0.25 or 0.50% sodium deoxycholate (TPGA-DC 0.25 or TPGA-DC 0.50). Cells sampled after no heating produced colony counts on TPGA-BS 0.85 or on TPGA-DC 0.50 that were no more than about 0.5 log lower than for unheated cell samples plated on TPGA. Cells heated at 50°C for 30 min produced colony counts on TPGA-DC 0.50 or on TPGA-BS 0.85 that were about 1.5 logs lower than on TPGA. Cells heated for 30 min and shifted to TPG broth at 37°C to allow resuscitation required about 2 h to regain tolerance to 0.85% BS. However, heated cells resuscitated on solid TPGA at 35°C before being challenged with overlays of TPGA-BS 0.85 or TPGA-DC 0.50 required 6 to 8 h on TPGA to regain tolerance to 0.85% BS or 0.50% DC. To regain tolerance to overlays of 0.15% BS or 0.25% DC, heated cells required resuscitation periods on TPGA of about 2 to 6 h, respectively. Cells heated in TPG broth and sampled after no heating produced colony counts on TPGA that were about 1.5 logs lower than for unheated cell suspensions, suggesting greater apparent injury when heat stressed in broth than in buffer. Our results indicate that heat-stressed S. flexneri cells apparently required a longer period of resuscitation on a solid, nonselective medium, by 2 to 4 h, to regain tolerance to a selective agent, BS or DC in our tests, than has been reported for other gram-negative bacteria.

Injured microorganisms are important in all aspects of food microbiology. Sublethal injury induced by exposure to treatments related to food processing, such as heat (10), often is demonstrated as a loss by the microorganism of one or more abilities to function characteristically under conditions that are satisfactory for untreated cells (1, 11–13).

Heat-induced cell injury of the following gram-negative bacteria has been studied in recent years: Vibrio parahaemolyticus (2, 6, 14), Salmonella typhimurium (4, 21, 22), and Yersinia enterocolitica (18; D. V. Pruitt and M. G. Johnson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, P3, p. 187).

Resuscitation treatments should ideally be applicable to all kinds of foods, organisms, injuries, and isolation techniques, since many factors which influence cell resuscitation are unknown (23). The time needed for the resuscitation of stressed bacterial cells may vary.

Since Shigella cells may be susceptible to heat injury, they may not be recovered from foods by using the selective media currently in use for the detection and isolation of this organism. To discourage growth of gram-positive bacteria, these media contain bile salts as a selective agent. Moreover, since Shigella cells can cause foodborne dysentery even when ingested in low numbers (3, 24), they could easily escape detection if they were injured and unable to grow in the presence of selective agents, thereby causing a significant error in the determination of the presence of Shigella cells present in food products.

To the best of our knowledge, no studies of heat injury of Shigella cells have been reported to date. The objective of this study was to investigate the sensitivity to and recovery of tolerance to bile salts of sublethally heated cells of a documented foodborne strain of Shigella flexneri.

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MATERIALS AND METHODS

Cultures. The S. flexneri strain (M4243) used in this study was obtained from A. D. O'Brien, Department of Bacterial Diseases, Walter Reed Army Medical Center, Washington, D.C. Culture identity was verified by using the API 20E Entero bacteriaceae System (Analytab Products, Plainview, N.Y.). Cultures were maintained on tryptic Phytone glucose agar (TPGA), which contained (per liter of distilled water): Trypsinase peptone (BBL Microbiology Systems, Cockeysville, Md.), 17.0 g; Phytone peptone (BBL), 3.0 g; sodium chloride, 5.0 g; dipotassium phosphate, 2.5 g; glucose, 2.5 g; agar (flame; Difco Laboratories, Detroit, Mich.) 15.0 g. When prepared without glucose or without agar, this medium was designated TPA or TPG broth, respectively.

Pour plates were made by pipetting 10 ml of sterile, tempered agar into each petri dish. In the overlay procedure (9, 19), 10 ml of medium was used as the overlay at the times indicated.

Appropriate amounts of bile salts no. 3 (BS; lot no. 632558; Difco) were added to TPGA before autoclaving to give the desired final concentrations (wt/vol) of 0.15% (TPGA-BS 0.15) or 0.85% (TPGA-BS 0.85). A 20% solution (wt/vol) of sodium deoxycholate (DC) was sterilized by vacuum filtration (pore size, 0.45 μm), and amounts were...
added to sterile, tempered TPGA to give final concentrations (wt/vol) of 0.25% (TPGA-DC 0.25) or 0.50% (TPGA-DC 0.50).

Heat injury. Cultures for heat stress were grown by inoculating 1 ml of a 10-ml overnight suspension into 99 ml of TPG broth in a Belco 250-ml Erlenmeyer shake flask. The flask was incubated at 37°C in a water bath shaker (model G76; New Brunswick Scientific Co., Inc., Edison, N.J.) at 200 rpm for 12 h to give cells which were in the early stationary phase of growth.

The cell suspension was transferred to a sterile 250-ml dilution bottle, and the cells were sedimented by centrifugation for 10 min at 13,200 × g at 37°C and washed twice with 0.1 M sodium phosphate buffer which was adjusted to pH 7.0 and tempered at 35°C. This buffer was used throughout this study and also served as the diluent except where noted. Control samples were taken at this time to determine the colony counts produced by unheated cells in the suspension. The cell suspension was heated in an 800-ml Kjeldahl flask in a water bath kept at 50 ± 0.1°C and was stirred by a magnetic bar. The suspension reach 50.0°C in about 5 min (determined by direct thermometer reading), and samples for enumeration were taken immediately (time zero) and 15 and 30 min thereafter.

Appropriate dilutions were spread plated on the above media and incubated at 35°C for 24 h before being counted. The difference in colony counts obtained on TPGA medium versus TPGA-BS or TPGA-DC media indicated the fraction of the heated cell population that became sensitive to these bile salts preparations. The heat-induced sensitivity of cells to bile salts is referred to as cell injury.

To measure the ability of heat-stressed cells to regain tolerance to bile salts, heat-stressed cells were harvested by centrifugation as described above and resuspended in the resuscitation broth, TPG broth, and incubated at 37°C with shaking at 200 rpm. At 1- or 2-h intervals over an 8-h period, 1-ml samples of suspension were withdrawn and the appropriate decimal dilutions were made and spread plated on the above media. Plates were incubated and counted as described above.

To measure cell resuscitation on agar media, the heat-stressed cells were harvested and spread plated as above, except that the 15-min heated sample was omitted. At the indicated times, cells were overlaid (9, 19) with the desired agar medium which had been tempered to 47°C, and the colonies were enumerated after the plates were incubated upright at 35°C for 48 h. All agar overlay procedures are symbolized by listing media in chronological order of use.

![Graph](image_url) **FIG. 1.** Survival curves for cells of *S. flexneri* heated at 50°C in 0.1 M sodium phosphate buffer, pH 7.0. Cells were plated at times indicated on TPGA (○), TPGA-BS 0.85 (△), or TPGA-DC 0.50 (□).
from left to right. For example, TPGA overlaid with TPGA-BS is written as TPGA/TPGA-BS.

Data are expressed as means of duplicate experiments with all samples plated in triplicate. The statistical significance of the means was determined by using the Duncan multiple range test (20).

RESULTS

Heat stress in 0.1 M sodium phosphate buffer. Survival curves for cells of S. flexneri M4243 heated in 0.1 M phosphate buffer at 50.0°C for 30 min are shown in Fig. 1. The colony count on TPGA, the control medium, was only about 0.6 log lower for cells heated for 30 min than for unheated cells. Cells heated for 30 min produced colony counts on TPGA-BS 0.85 or TPGA-DC 0.50 that were about 1.5 logs lower than the counts on the control TPGA medium.

Cells heated on 0.1 M phosphate buffer and then resuspended in TPG broth required 6 h to regain their tolerance to BS (Fig. 2). Heated cells suspended in TPG broth for 1, 2, or 3 h produced significantly lower colony counts (P ≤ 0.05) on TPA-BS 0.85 or TPGA-BS 0.85 media than on TPA (TPGA minus glucose) or TPGA media. A suspension of unheated cells stored in TPG broth, when sampled over an interval of 1 to 8 h and plated on the above control media, showed no significant increase in colony counts (data not shown). Both the shape of the recovery curve and the absence of an increase in the TPG counts (Fig. 2) indicated that the cellular event being measured was a resuscitation of preexisting cells, not growth of new cells.

The sensitivities of cells heated in phosphate buffer to DC and BS were compared by using the overlay procedure (Fig. 3). Heated cells required at least 6 h of resuscitation on the nonselective basal layer, TPGA, before regaining tolerance to either 0.85% BS or 0.50% DC. Heated cells plated directly on a basal layer of selective agar and then overlaid at 1 to 8 h with the homologous selective agar did not resuscitate. On these plates with double layers of selective media, the log colony counts remained constant at 5.9 to 6.0 or 5.4 to 5.6 over the 8-h period and significantly lower (P ≤ 0.05) than for cells allowed to first resuscitate on the nonselective agar medium.

Studies with heated cells allowed to resuscitate on TPGA before exposure to TPGA with 0.15% or 0.85% BS (Fig. 4) or to TPGA with 0.25% or 0.5% DC (Fig. 5) indicated that these two agents at the lower of the two test concentrations were substantially less inhibitory to heat-stressed cells of this organism. These data indicate that stressed cells required resuscitation times of only 0 to 2 h to tolerate overlays with 0.15% BS or 0.25% DC but 6 to 8 h of resuscitation to tolerate overlays with 0.85% BS or 0.5% DC.

Heat stress in broth medium. To stimulate the effect of food environment on the survival of thermally stressed cultures, cells of S. flexneri were heated for 30 min at 50.0°C in TPG broth (Fig. 6). In contrast to previous results for cells heated in buffer, colony counts for cell suspensions heated in TPG broth and then plated on and overlaid with the control medium (TPGA/TPGA) were more than 1.5 logs lower than colony counts for the unheated cells plated by the same procedure on the same control medium (Fig. 6). Heated cells produced colony counts on TPGA/TPGA-DC 0.30 agar overlay procedure that were significantly lower at 0 to 4 h (P
yielding a contamination with bile would be difficult. Furthermore, a period of 4 to 8 h is needed before as many as 50% of the stressed cell population regains tolerance to the bile salts compounds. Our results suggest that media intended for enumeration of heat-stressed Shigella cells should be formulated to contain less than 0.85% BS or 0.50% DC.

The resuscitation period of 4 to 8 h we observed for S. flexneri cells is considerably longer than the 1- to 2-h resuscitation period reported (8, 16–18) for freeze- or chill-stressed coliforms from seafood and dairy products. Moreover, cells of Campylobacter jejuni heat injured at 46°C in buffer were recently reported (15) to require 4 h to regain tolerance to bile salts.

For cells heated in broth, the control counts on TPGA/TPGA (Fig. 6) were significantly lower than for cells heated in buffer. The cells heated in broth, when allowed to resuscitate on the nonselective agar before challenge with the bile salts, were apparently less able to regain tolerance to these salts than were the cells heated in buffer. Our results are consistent with reports (7, 16) for heat-stressed cells of Salmonella typhimurium, in which lower counts were obtained in more nutritionally rich media. A recent report (I. J. Mehlman and B. A. Wentz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, P3, p. 200) indicated that Escherichia coli strains incubated at 44°C in a rich 2% tryptone broth medium were not quantitatively recovered if as little as 0.15% BS were present in the medium. Our results, coupled with the above reports, point to the need for more work to establish the maximal levels of BS or DC which can be tolerated by heat-stressed cells of gram-negative bacteria of concern in foods.

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LITERATURE CITED


TO OVERLAY

Fig. 6. Resuscitation of S. flexneri cells heated in broth and plated by the overlay procedure on TPGA medium with or without BS or DC. Cells were heated for 30 min in TPG broth, diluted in TPG broth, plated on the basal layer of nonselective or selective agar medium, and incubated at 35°C for 2 to 8 h before the overlay medium was added. Symbols: •, TPGA/TPGA; △, TPGA/TPGA-BS 0.85; □, TPGA/TPGA-DC 0.50; ▲, TPGA-BS 0.85/TPGA-BS 0.85; ▼, TPGA-DC 0.50/TPGA-DC 0.50; —, unheated control cells; ——, cells heated for 30 min.

\[ P \leq 0.05 \] but not at 6 to 8 h (\( P \leq 0.05 \)) of resuscitation compared with those of the controls on TPGA/TPGA. Counts on TPGA/TPGA-BS 0.85 remained significantly lower (\( P \leq 0.05 \)) even if 8 h of resuscitation were allowed before the cells were overlaid with BS-containing medium TPGA-BS 0.85.

DISCUSSION

This results of this study show that cells of S. flexneri M4243, when sublethally heat stressed at 50°C for 30 min, become sensitive to 0.85% BS or 0.50% DC. Moreover, such heat-stressed cells do not regain their tolerance to these concentrations of bile salts until the cells are allowed to resuscitate on a nonselective agar such as TPGA for about 6 h.

Several commercial media recommended (5) for isolation and enumeration of enteric bacteria contain BS in the following concentrations: MacConkey agar, 0.15%; Salmonella-Shigella agar, 0.85%; and Hektoen Enteric Agar, 0.9%. Similarly, other media contain DC as a selective agent: Deoxycholate Citrate agar, 0.50%; and XLD agar, 0.25%. Our results indicate that BS at 0.85% or DC at 0.50% in TPGA interfered with the enumeration of heat-stressed cells of S. flexneri.

The data presented here suggest that for food naturally contaminated with 100 sublethally heated S. flexneri cells per g—a number sufficient to produce foodborne illness (5)—it would be difficult to detect these cells at this low level of contamination with bile salts-containing media, thereby yielding a false-negative result.

Data presented here indicate that cells of S. flexneri heat stressed at 50.0°C for 30 min require 2 to 4 h of resuscitation on a nonselective agar such as TPGA before they start to regain tolerance to BS or DC. Furthermore, a period of 4 to 8 h is needed before as many as 50% of the stressed cell population regains tolerance to the bile salts compounds. Our results suggest that media intended for enumeration of heat-stressed Shigella cells should be formulated to contain less than 0.85% BS or 0.50% DC.

The resuscitation period of 4 to 8 h we observed for S. flexneri cells is considerably longer than the 1- to 2-h resuscitation period reported (8, 16–18) for freeze- or chill-stressed coliforms from seafood and dairy products. Moreover, cells of Campylobacter jejuni heat injured at 46°C in buffer were recently reported (15) to require 4 h to regain tolerance to bile salts.

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