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Thermal Injury of *Yersinia enterocolitica*

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Procedures were developed to evaluate thermal injury to three strains of *Yersinia enterocolitica* (serotypes 0:3, 0:8, and 0:17). Serotype 0:17 (atypical strain) was more sensitive to bile salts no. 3 (BS) and to sublethal heat treatment than the typical strains, 0:3 and 0:8. When the 0:3, 0:8, and 0:17 serotypes were thermally stressed in 0.1 M PO_4 buffer, pH 7.0, at 47°C for 70, 60, and 12 min, respectively, greater than 99% of the total viable cell population was injured. Injury was determined by the ability of cells to form colonies on brain heart infusion (BHI) agar, but not on Trypticase soy agar (TSA) plus 0.6% BS for serotypes 0:3 and 0:8 and TSA plus 0.16% BS for 0:17. Heat injury of serotype 0:17 cells for 15 min in 0.1 M PO_4 buffer caused an approximate 1,000-fold reduction in cell numbers on selective media as compared with cells heated in pork infusion (PI), BHI broth, and 10% nonfat dry milk (NFDM). The extended lag and resuscitation period in BHI broth was 2.5 times greater for 0:17 cells injured in 0.1 M PO_4 than for cells injured in BHI or PI. The rate and extent of repair of *Y. enterocolitica* 0:17 cells in three recovery media were directly related to the heating menstruum used for injury. The use of metabolic inhibitors demonstrated that ribonucleic acid synthesis was required for repair, whereas deoxyribonucleic, cell wall, and protein synthesis were not necessary for recovery of 0:17 cells injured in 0.1 M PO_4 buffer, BHI, or PI. Inhibition of respiration by 2,4-dinitrophenol slowed repair only for 0:17 cells injured in 0.1 M PO_4 buffer, not for cells injured in PI or BHI.

Yersinia enterocolitica and *Y. enterocolitica*-like organisms (atypical strains) have been isolated from a wide variety of foods as well as from environmental sources. In an extensive European survey, Leistner et al. (43) identified *Y. enterocolitica* in chicken meat, pork, and beef. Strains have been isolated from vacuum-packaged beef, lamb, and dark firm meat (pH $\geq$ 6.5) held under refrigeration (20, 26, 54), raw and pasteurized milk (32, 52), cheeses (51), mussels, ice cream, bananas, fish, ham, and sausage (41). They have also been isolated from pond and well water (11), streams, lakes (28), and drinking water (39).

Only one foodborne outbreak caused by *Y. enterocolitica* has been documented (14), although food sources were implicated in other outbreaks (15, 29, 59). In 1976, an illness occurred among school children in Oneida County, New York (5). Chocolate milk was determined to be the common vector of transmission. Serotype 0:8 was isolated from an unopened carton of the milk and from the hospitalized children. Thirty-three children were hospitalized for suspected appendicitis, and 13 appendectomies were performed.

Many treatments used in food processing and combinations of treatments may induce sublethal damage to cells or spores (12). In addition, the production of bacterial end products such as organic acids in foods may cause sensitive bacterial cells to become stressed or injured. Therefore, unprocessed as well as processed food products have the potential of harboring sublethally damaged microorganisms.

Many cellular and molecular modifications may occur in injured cells. The extent of sublethal damage and the mechanism of injury vary and may be related to the stress condition. Various manifestations of damage have been reported (6, 15, 18, 21, 22, 27, 33, 37, 44, 48, 49, 57). Also, more than one damaged site may occur in a bacterial cell for a particular type of sublethal treatment (31, 37).

The ability to enumerate stressed bacterial cells in a food product is extremely important. Sensitivity to selective agents incorporated into isolation media appears to be a major characteristic of injured bacteria (12, 19). Since they cannot repair in the selective environment (47), they fail to multiply and consequently remain undetected in the selective media. Therefore, selective media may not be suitable for enumeration of microorganisms in processed foods. Damage to the cytoplasmic membrane or cell envelope, causing increased permeability, could be one

reason for greater sensitivity of injured cells to selective agents (12).

Many investigators (12, 21, 27, 33, 47-50) have emphasized the importance of repairing or resuscitating injured bacterial cells before the cells are exposed to selective compounds. Sublethally stressed microorganisms contain altered growth characteristics (19). Resuscitation of the injured bacterial cells occurs during the extended lag phase (22, 37, 48, 57). However, the time required for resuscitation of injured microorganisms depends on the nature of the sublethal treatment, the microbial species, the selective media used for enumeration, and the chemical composition and incubation conditions of the restoration media (47). Special treatments or conditions are, therefore, required to return the cells to their normal state (12). Injured cells are able to repair and multiply in a nonselective medium (12, 45, 48). Recently, however, results indicate that injured microorganisms may repair sublethal damage more readily in simple, less nutritious media (minimal media) than in complex media (34, 35). The enrichment medium used widely for isolation of *Y. enterocolitica* from foods has been phosphate-buffered saline at neutral pH incubated at 4°C for 14 to 28 days. However, the cold enrichment has not been evaluated for its recoverability of injured *Y. enterocolitica* cells.

There were three primary purposes in these studies: (i) evaluation of media and conditions for thermal injury of *Y. enterocolitica*; (ii) comparison of various recovery media and conditions of repair; and (iii) analysis of the mechanisms involved in thermal injury and recovery of *Y. enterocolitica* through the use of a variety of metabolic inhibitors.

MATERIALS AND METHODS

Bacterial strains. The three *Y. enterocolitica* strains used in this investigation were serotype 0:8 (ATCC 27739), isolated from stream water in the United States; 0:3 (obtained from H. H. Mollaret, Pasteur Institute, Paris), a human isolate from Europe; and 0:17 (atypical strain from Edward J. Bottone, Mt. Sinai Hospital, New York), a clinical isolate from the United States. Stock cultures were maintained on brain heart infusion (BHI; Difco Laboratories) slants and stored at 4°C.

Media and diluent. BHI broth was prepared according to manufacturer's specifications. Trypticase soy agar (TSA; BBL Microbiology Systems) plus bile salts no. 3 (BS; Difco) and BHI agar were used as assay media for the enumeration of injured cells. BS powder was dissolved in distilled water at a 10% (wt/vol) concentration on the day of use. The stock solution was sterilized by filtration through a 0.45-nm membrane. Stock solutions were stored no longer than 1 week at 4°C. Volumes of BS stock solution and predetermined volumes of TSA were mixed aseptically to obtain a BS concentration of 0.16 or 0.6%. The media were poured into sterile petri plates and allowed to dry at room temperature for 24 to 48 h before use.

Pork infusion (PI) broth was prepared with a slight modification of the procedure used by Andersen (2). Frozen special lean pork loins were thawed overnight at 10°C. Excess fat was trimmed, and the loins were ground through a 0.25-inch (ca. 0.6-cm) plate. The ground pork was added to 9 liters of distilled water (500 g/liter) and steamed to a slow boil in a covered steam jacket kettle for 1 h with intermittent stirring. After boiling, the contents were strained through a stainless-steel colander to remove large chunks of meat. The filtrate was refrigerated at 4°C for 24 h, and the top fat layer was removed with a stainless-steel spatula. The infusion was heated to liquify the gel and strained through four layers of cheesecloth to remove small meat fragments. The infusion was then filtered twice through clarifying filter sheets. The final filtrate was slightly turbid. The PI was sterilized by autoclaving for 15 min at 121°C. The final pH was 6.5 with a water activity of 0.98. Excess PI was stored at −34°C in 2-liter polypropylene containers until use.

A 10% nonfat dry milk (NFDM) solution was formulated by reconstituting spray dried milk solids. The 10% NFDM solution was thoroughly mixed and autoclaved at 121°C for 7 min with a final pH of 6.6. Slight browning was observed.

A chemically defined glucose salts medium (GSM) was used for recovery of injured cells. It contained: $MgSO_4 \cdot 7H_2O$, 0.15 g; $K_2HPO_4 \cdot 3H_2O$, 4.0 g; KH_2PO_4, 2.0 g; $(NH4)_2SO_4$, 1.0 g; sodium citrate·$2H_2O$, 0.1 g; and glucose, 4.0 g; in 1 liter of distilled water. A 10% glucose solution was autoclaved separately at 121°C for 15 min and added aseptically to the sterile basal salts medium at the desired concentration. The pH of the GSM was 7.0. The diluent used in all experiments was 0.1% peptone water.

Cultural and harvesting conditions. Cells from a BHI agar slant were transferred aseptically to 10 ml of BHI broth and incubated at 25°C for 24 h. A 0.5-ml volume was inoculated into 125 ml of BHI broth in a 250-ml Erlenmeyer flask and incubated under static conditions at 25°C for 18 to 24 h. This procedure yielded a bacterial culture of approximately 10^9 cells/ml, which corresponded to a 0.96 to 1.10 absorbancy reading (1.91-cm-diameter tube) at 420 nm. Duplicate 10-ml samples of the culture were centrifuged in the cold (4 to 8°C) at 4,350 × *g* for min. The supernatant liquid was discarded, and the pellet was resuspended in 10 ml of the heating menstruum and centrifuged as described above. The washed pellet was resuspended in 5 ml of the heating menstruum, and the duplicate cell suspensions were mixed and stirred in a Vortex mixer.

Thermal stress. Cells were subjected to thermal stress by using the shaker flask method. Five-milliliter volumes of the washed cell suspension were inoculated into two 250-ml Erlenmeyer flasks, each containing 95 ml of the corresponding heating menstruum to give 10^8 cells/ml. The heating menstruums were BHI broth, PI, 0.1 M PO_4 buffer, pH 7.0, and 10% NFDM. One set of cultures (47 ± 0.2°C) was heated in a water bath equipped with a shaker (120 rpm) to provide

uniform temperature and cell suspension. A second set of cultures was chilled in an ice bath and manually shaken once every 2 to 3 min. Preliminary experiments showed that the times that the rate of injury leveled off for 47°C heat injury were 12, 60, and 70 min for serotypes 0:17, 0:8, and 0:3, respectively. At the termination of the heating period, the cells were either centrifuged or directly added to the recovery media.

Recovery procedures. After the desired heating time, 2-ml volumes of the heated suspension were added directly to 98 ml of BHI broth tempered at 25°C, to give 10^6 viable cells/ml. When metabolic inhibitors were used, 1 ml of the sterile antimicrobial stock solution was added to 97 ml of BHI broth in a 250-ml Erlenmeyer flask just before the addition of the heated suspension. One-milliliter volumes of sterile solvent solution without the metabolic inhibitor were added to 97 ml of BHI broth, which served as the control.

In experiments comparing the recovery curves of injured cells in BHI broth, GSM, and sterile distilled water, 10 ml from the heated suspension was immediately added to a sterile centrifuge to be immersed in an ice bath. The centrifuge tube was swirled to rapidly cool the cell suspension. The contents were centrifuged 5 min at 12,100 × *g*. The pellet was resuspended in 10 ml of chilled sterile distilled water, and 2-ml volumes were immediately added to 98 ml of BHI broth, GSM, and sterile distilled water tempered at 25°C, to give 10^6 viable cells/ml. All recovery media were incubated at 25°C.

Enumeration procedure. The spread plate counting technique was used to determine the amounts of thermal injury and recovery. A 1-ml volume was removed from the heating menstruums or recovery media at various intervals and serially diluted in 0.1% peptone water blanks. Samples of 0.1 ml were spread plated in triplicate onto BHI agar and TSA containing 0.6 and 0.16% BS. The plate cultures were incubated at 25°C for 48 h. Only plates containing 30 to 300 colonies were counted. Preliminary experiments showed that TSA plus 0.6% BS proved best for enumerating uninjured cells for serotypes 0:3 and 0:8, whereas only 0.16% BS in TSA was needed to enumerate serotype 0:17 uninjured cells. Since no significant difference between BHI agar and TSA plates was calculated for the recovery of total viable bacterial population, BHI agar plates were used to estimate the total number of viable cells. The number of injured cells equaled the counts in BHI agar plates minus the counts on TSA plus 0.6 or 0.16% BS plates. The number of uninjured cells equaled the bacterial counts on the TSA plus BS plates.

Inhibitors. Minimum inhibitory concentrations (MIC) of five metabolic inhibitors against *Y. enterocolitica* 0:17 were determined in BHI broth by the tube dilution technique (53). Inhibitors included chloramphenicol (crystalline), penicillin G (potassium salt; 1 U 0.62 g), rifampin (crystalline), nalidixic acid (powder), and 2,4-dinitrophenol (grade II, 90 to 95% pure, contains 15 to 20% water). Stock solutions of all of the above except rifampin, nalidixic acid, and 2,4-dinitrophenol were prepared in distilled water. Rifampin (4.8 mg) was dissolved in 1 ml of dimethyl sulfoxide (spectrophotometric grade), and the solution was diluted to 6.7% dimethyl sulfoxide with 14 ml of distilled water. A 2,4-dinitrophenol solution was prepared by dissolving 23.2 mg of this compound in 0.1 ml of absolute ethanol, diluting to 0.67% ethanol with 14.9 ml of distilled water, and heating at 90°C in a water bath for 10 min. Nalidixic acid (9.6 mg) was dissolved in 0.2 ml of 5 N NaOH and diluted to 0.07 N NaOH with 14.8 ml of distilled water. The concentrations of each metabolic inhibitor stock solution were 320 μg/ml for chloramphenicol and rifampin and 640, 5,120, and 1,546 μg/ml for nalidixic acid, penicillin G, and 2,4-dinitrophenol, respectively. These solutions were filter sterilized through a 0.45-nm membrane filter, dispensed into tubes wrapped in aluminum foil, and stored at 4°C for 2 days before use. One-milliliter volumes of serial twofold dilutions in distilled water of each solution were added to duplicate 8-ml 1.1× strength BHI broth. A 1-ml volume (10^6 viable cells) of a diluted *Y. enterocolitica* serotype 0:17 culture (prepared as described above) was pipetted into the BHI broth containing the metabolic inhibitors. Appropriate duplicate controls were prepared for each metabolic inhibitor. The tubes were incubated at 25°C for 24 h. The MIC was defined as the lowest antimicrobial concentration in which no visible bacterial growth was observed.

For experiments involving the effect of metabolic inhibitors on recovery, inhibitors were used at the MIC for *Y. enterocolitica* serotype 0:17 as determined in this investigation. The stock solutions (100-fold concentration) and controls of each metabolic inhibitor were prepared as described above and stored at 4°C for 1 day before use. Heat-stressed cell suspensions (2 ml) were pipetted into 98 ml of BHI broth with and without the metabolic inhibitor as described in the recovery procedures section. Serial dilutions of the recovery medium prevented interference of the metabolic inhibitors with growth of *Y. enterocolitica* 0:17 cells on the various assay media.

Statistical analysis. Data from all injury and recovery experiments were analyzed statistically by different methods. Data from the injury experiments involving serotypes 0:8 and 0:3, all recovery curves, and the injury of *Y. enterocolitica* 0:17 cells in various heating menstruums were analyzed statistically by transforming the numbers to $\log_{10}$ bacterial counts per milliliter and taking the geometric means of triplicate samples. For injury data of serotype 0:17 cells heated in various menstruums, the mean $\log_{10}$ bacterial count per milliliter enumerated on BHI agar at time zero was arbitrarily assigned 0.00 to obtain a single starting point. Subsequent mean $\log_{10}$ counts per milliliter recovered on BHI agar and TSA plus 0.16% BS were subtracted from the actual mean $\log_{10}$ count on the initial BHI agar. Numbers of nonviable cells were calculated by subtracting the initial mean $\log_{10}$ bacterial count on BHI from the mean $\log_{10}$ bacterial counts on BHI agar at designated times during heating.

Data from the studies comparing the recovery of heat-stressed and unstressed *Y. enterocolitica* cells on BHI agar, TSA, and TSA with BS incubated for 48 and 96 h were analyzed statistically by the percent recovery Student *t*-test and by the one-way analysis

of variance using Dunnett's test (17) for multiple comparison with control.

RESULTS

Enumeration of unstressed and heat-stressed *Y. enterocolitica* cells. Several solid plating media and selective agents were evaluated for their ability to enumerate normal and heat-stressed *Y. enterocolitica* cells. Various concentrations of the selective agents NaCl, BS, and sodium deoxycholate (Difco) were incorporated into BHI agar, TSA, TSA plus 0.5% yeast extract, xylose lysine agar base (Difco) with 1% peptone (38), and MacConkey agar (Difco). Of the selective media tested, TSA plus BS proved best for estimating stressed *Y. enterocolitica* 0:17, 0:8, and 0:3 cells when heated at 47°C in 0.1 M PO_4 buffer, pH 7.0. For example, when samples of heated cell suspensions are plated on a nonselective medium, BHI agar, and TSA plus BS, the difference in counts between the former and the latter yields an estimate of the number of heat injured cells.

When chilled (4°C) and heated (47°C) cell suspensions of *Y. enterocolitica* 0:17, 0:3, and 0:8 were plated on two nonselective media, i.e., BHI agar and TSA, as well as the selective medium, TSA plus BS, marked differences in viable counts were observed (Table 1). Counts on BHI agar plates that were incubated for 48 h arbitrarily represented a 100% recovery of heated or chilled *Y. enterocolitica* cells. There were no significant ($P > 0.05$) differences in counts with time or plating media for chilled (normal) *Y. enterocolitica* 0:17, 0:8, and 0:3 cells (Table 1). In contrast, although there were no significant ($P > 0.05$) differences between the recovery of cells on BHI and TSA plates, colony counts were significantly reduced on TSA plus BS plates. Since colonies were larger and, therefore, more easily counted, BHI agar was selected for subsequent enumeration of heat-injured *Y. enterocolitica*.

TABLE 1. *Comparison of various media for the recovery of normal and thermally injured Y. enterocolitica serotypes 0:17, 0:8, and 0:3*[a]

Serotype	Plating medium[b]	% of initial BHI count			
		Chilled		Heated	
		48 h	96 h	48 h	96 h
0:3	BHI agar	100.0[c]	101.1	100.0[c]	102.3
	TSA	100.0	101.6	97.1	98.5
	TSA + 0.6% BS	99.5	100.0	0.2[d]	0.3
0:8	BHI agar	100.0	100.0	100.0	100.0
	TSA	98.4	100.3	84.4	85.9
	TSA + 0.6% BS	89.3	89.8	0.05	0.05
0:17	BHI agar	100	100.0	100.0	100.9
	TSA	94.5	95.9	107.5	107.7
	TSA + 0.16% BS	94.5	97.3	0.1	0.2

[a] Cells were thermally injured by heating in 0.1 M phosphate buffer, pH 7.0, at 47°C for 60 (serotype 0:8), 70 (serotype 0:3), and 12 (serotype 0:17) min.

[b] Plating media were incubated at 25°C for 48 and 96 h. Data were based on average of three experiments with triplicate plates being used in each experiment. The inoculum contained approximately 10^7 cells.

[c] Dunnett's test for multiple comparison with a control was used to calculate the comparison between BHI agar plate counts incubated for 48 h and TSA plate counts incubated for 48 and 96 h, and TSA plus BS counts incubated for 48 and 96 h for heated and normal cells. The number of chilled and heated cells recovered on BHI agar plates after 48 h of incubation was considered a 100% recovery.

[d] Student's *t*-test was used to calculate the significant differences between the enumeration of heated cells on TSA plus BS plates incubated for 48 and 96 h.

Thermal injury. Thermal injury curves for *Y. enterocolitica* serotypes 0:3 and 0:8 heated at 47°C in 0.1 M PO_4 buffer, pH 7.0, are presented in Fig. 1. Although a small percentage of death was evident, the total viable counts on BHI agar remained fairly constant during the heating time of 100 min. During the first 60 min of heating, the rate of injury was rapid for serotype 0:3 and 0:8 cells. During subsequent heating, rate of injury decreased. The extent of injury to serotype 0:3 cells was less than that of 0:8 cells. In addition, at the end of the 100-min heating at 47°C in 0.1 M PO_4 buffer, 2.50 and 3.60 log number of bacterial per ml of the total viable cell population (approximately 10^8 cells/ml) for serotypes 0:3 and 0:8, respectively, failed to recover as evidenced by inability to grow on TSA plus 0.6% BS plates (Fig. 1).

Thermal injury curves for *Y. enterocolitica* serotype 0:17 cells heated at 47°C in different menstruums are illustrated in Fig. 2. *Y. enterocolitica* serotype 0:17 cells displayed an initially rapid inability to grow on the selective medium similarly to serotypes 0:3 and 0:8 cells (Fig. 1).

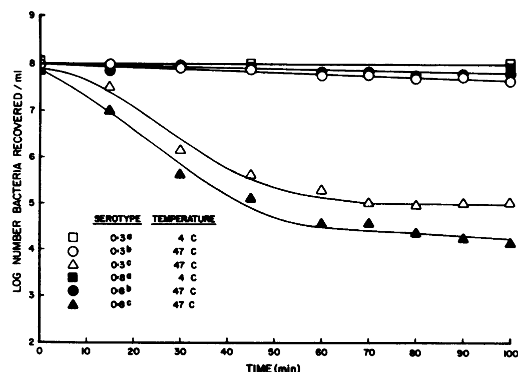

FIG. 1. *Thermal survival curves of Y. enterocolitica serotype 0:3 and 0:8 cells heated in PO_4 buffer menstruum. Cells were heated in 0.1 M PO_4 buffer, pH 7.0, at 47°C. Controls used the chilled PO_4 menstruum. a, BHI agar and TSA plus 0.6% BS; b, BHI agar; c, TSA plus 0.6% BS.*

However, this rapid rate of injury for serotype 0:17 cells occurred within only 8 to 10 min of heating in the different menstruums investigated. During the 16-min heating period in the four menstruums, the total viable serotype 0:17 count on BHI agar plates remained fairly constant, although some death was evident.

The rate and extent of injury for *Y. enterocolitica* 0:17 cells varied depending on the type of heating menstruum used (Fig. 2). The fastest rate and greatest extent of injury for 0:17 cells heated at 47°C occurred in 0.1 M PO_4 buffer, pH 7.0. In this menstruum, at the end of 16 min of heating, 3.30 log number of bacteria per ml of a total viable cell population (approximately 10^7 cells/ml) was injured or failed to grow on TSA plus 0.16% BS plates. When serotype 0:17 cells were heated at 47°C in PI, BHI broth, or 10% NFDM, the rates of injury during the initial 8 min of heating were similar, but considerably less when the cells were heated in 0.1 M PO_4 buffer. Furthermore, after the 16-min heating period, only 1.51, 1.04, and 1.07 log number of bacteria per ml of the total viable 0:17 cell population (approximately 10^8 cells/ml) were injured when heated in PI, BHI broth, and 10% NFDM menstruums, respectively (Fig. 2). Since heating at 47°C of serotype 0:17 cells in BHI or 10% NFDM displayed similar injury curves, further investigations using 10% NFDM were eliminated.

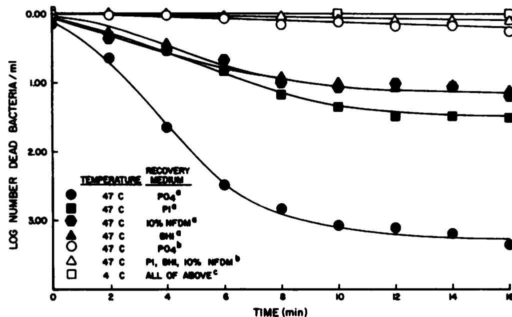

FIG. 2. *Thermal injury curves of Y. enterocolitica serotype 0:17 cells heated in various menstruums. Cells were heated in 0.1 M PO_4 buffer, pH 7.0, PI, BHI broth, and 10% NFDM at 47°C. Controls utilized the chilled menstruums. a, TSA plus 0.16% BS; b, BHI agar; c, BHI agar and TSA plus 0.16% BS.*

Recovery and growth in BHI broth of heat-injured serotype 0:17 cells. During resuscitation from thermal stress, a frequent manifestation is an extended lag phase (12, 19, 37, 57). This increased lag and the resuscitation of heat-injured *Y. enterocolitica* serotype 0:17 cells heated in 0.1 M PO_4 buffer, pH 7.0, BHI broth, and PI menstruums are presented in Fig. 3. Resuscitation was characterized by the ability of injured 0:17 cells to recover their tolerance to the TSA plus 0.16% BS medium. After heating for 12 min in the three heating menstruums, 0:17 cells were directly added to BHI broth incubated at 25°C, in which resuscitation occurred immediately. The length of the extended lag phase and the slope of the resuscitation curves of heat injured 0:17 cells were dependent on the heating menstruums used. When serotype 0:17 cells were heat injured at 47°C in 0.1 M PO_4 buffer for 12 min and subsequently inoculated into BHI broth, the length of the extended lag phase and resuscitation curve for injured cells was 5 h (Fig. 3A). In contrast, when 0:17 cells were heat injured in BHI or PI, the extended lag

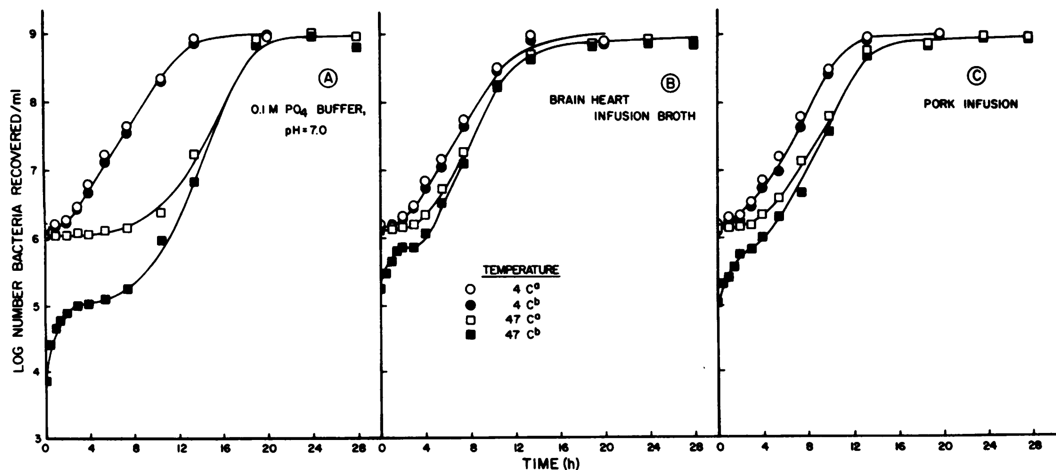

FIG. 3. *Recovery and growth of injured Y. enterocolitica (serotype 0:17) cells heated in the three menstruums. Cells were heated in (A) 0.1 M PO_4 buffer, pH 7.0, (B) BHI broth, and (C) PI for 12 min at 47°C. Controls used cells chilled for 12 min in the three menstruums. After heating and chilling, 2-ml volumes were added to 98 ml of BHI broth which was incubated at 25°C. a, BHI agar; b, TSA plus 0.16% BS.*

phase and resuscitation curves for injured cells occurred for only 2 h (Fig. 3B, C). Moreover, resuscitation was incomplete; i.e., counts on BHI agar plates were greater than counts on TSA plus BS plates. Cells chilled in the three menstruums displayed a lag phase in BHI broth of <1 h. Further, both the absence of an increase in BHI agar counts during the extended lag phase or recovery periods and the shape of the resuscitation curves indicated that recovery was not due to cell growth. When completely recovered, the heat-injured 0:17 cells commenced to grow at the same rate as unheated cells and reached the same total viable population in BHI broth incubated at 25°C.

Recovery of heat-injured 0:17 cells in GSM, BHI broth, and water. The rate and extent of resuscitation of 47°C heat-injured *Y. enterocolitica* 0:17 cells incubated in the recovery media (BHI broth, GSM, and water) at 25°C were directly related to the type of heating menstruum (Fig. 4). At various time intervals, 1-ml samples were removed from each recovery medium and plated on BHI agar and TSA plus 0.16% BS. Increased counts on TSA plus BS indicated resuscitation. Control chilled cells were analyzed in the same manner. Viable counts for chilled cells suspended in sterile distilled water remained constant irrespective of the plating medium, whereas cells incubated in BHI broth and GSM initiated multiplication after 30 min and 1 h, respectively (Fig. 4A). Resuscitation of heat-injured 0:17 cells in PO_4 buffer increased rapidly at similar rates in GSM and BHI broth for 2 h and then remained constant (Fig. 4B). After 2 h, approximately 1.00 log number of bacteria/ml resuscitated in BHI broth, but only 0.75 log number of bacteria/ml resuscitated in GSM. Furthermore, injured cells suspended in water did not resuscitate. Indeed, progressive death (reduction in BHI agar counts) and injury (decrease in TSA plus 0.16% BS counts) occurred.

Resuscitation occurred in GSM, BHI broth, and distilled water for cells heat-stressed in BHI for 12 min (Fig. 4C). Injured cells suspended in GSM exhibited the fastest rate of resuscitation. After 1 and 2.5 h, rates of resuscitation of injured cells suspended in water and BHI broth, respectively, decreased. In summary, after 3.5 h of incubation, 1.43, 1.20, and 0.89 log number of bacteria per ml of 0:17 cells injured in the BHI heating menstruum resuscitated when suspended in GSM, BHI broth, and distilled water, respectively.

Rapid resuscitation of serotype 0:17 cells heated in PI for 12 min at 47°C occurred when incubated in GSM and distilled water (Fig. 4D). After 1 h, 1.21 and 1.08 log number of bacteria per ml of the injured cells were resuscitated in GSM and distilled water, respectively. With a further incubation time, no increase in the rate of resuscitation of injured cells occurred. Resuscitation, on the other hand, occurred at a slower and linear rate when BHI broth was used as the recovery medium. After 3.5 h, the number of cells injured in the PI menstruum that were resuscitated in GSM and BHI broth was the same (approximately 1.40 log number of bacteria/ml), whereas only 1.17 log number of bacteria per ml of the injured cells resuscitated in distilled water.

MIC and effects of metabolic inhibitors on recovery of 0:17 cells. MIC values of 1, 2, and 8 μg/ml for nalidixic acid, chloramphenicol, and rifampin, respectively, were obtained for *Y. enterocolitica* 0:17 when incubated at 25°C in BHI broth (data not presented). However, these cells were more resistant to the action of penicillin G and 2,4-dinitrophenol, as indicated by MIC values of 128 and 155 μg/ml, respectively (data not presented). Metabolic inhibitors at corresponding MIC values were added to the BHI recovery medium to elucidate some of the biosynthetic processes involved during resuscitation of injured *Y. enterocolitica* 0:17 cells. When cells injured in 0.1 M PO_4 buffer, BHI broth, and PI were incubated in BHI recovery medium with or without choramphenicol, nalidixic acid, or penicillin G, rates of resuscitation were similar (data not presented). Nevertheless, nalidixic acid caused death to 0:17 cells thermally injured in BHI broth and PI. The lethal effects of these three antibiotics on injured *Y. enterocolitica* 0:17 cells occurred after resuscitation. Therefore, their effects on resuscitation of heat-injured cells were investigated. Since chloramphenicol, nalidixic acid, and penicillin G specifically inhibit bacterial protein synthesis (23), deoxyribonucleic acid (DNA) synthesis (16), and cell wall synthesis (7), respectively, it is evident that these biosynthetic processes were not required for resuscitation of heat-injured 0:17 cells. Rate and extent of resuscitation of 0:17 cells heated in 0.1 M PO_4 buffer and BHI broth and recovered in the presence of rifampin (8 μg/ml) were adversely affected (Fig. 5A, B). Cells heated for 12 min in phosphate buffer and recovered for 3 h in BHI broth showed resuscitation counts of 0.67 and 0.29 log number of bacteria/ml in the absence and presence of rifampin, respectively (Fig. 5B). In contrast, rifampin completely inhibited the resuscitation of the injured cells heated in the PI menstruum (Fig. 5C). Since this antibiotic is a potent inhibitor of bacterial ribonucleic acid (RNA) synthesis by binding irreversibly to the DNA-dependent RNA polymerase (58), *Y. enterocolitica* sero-

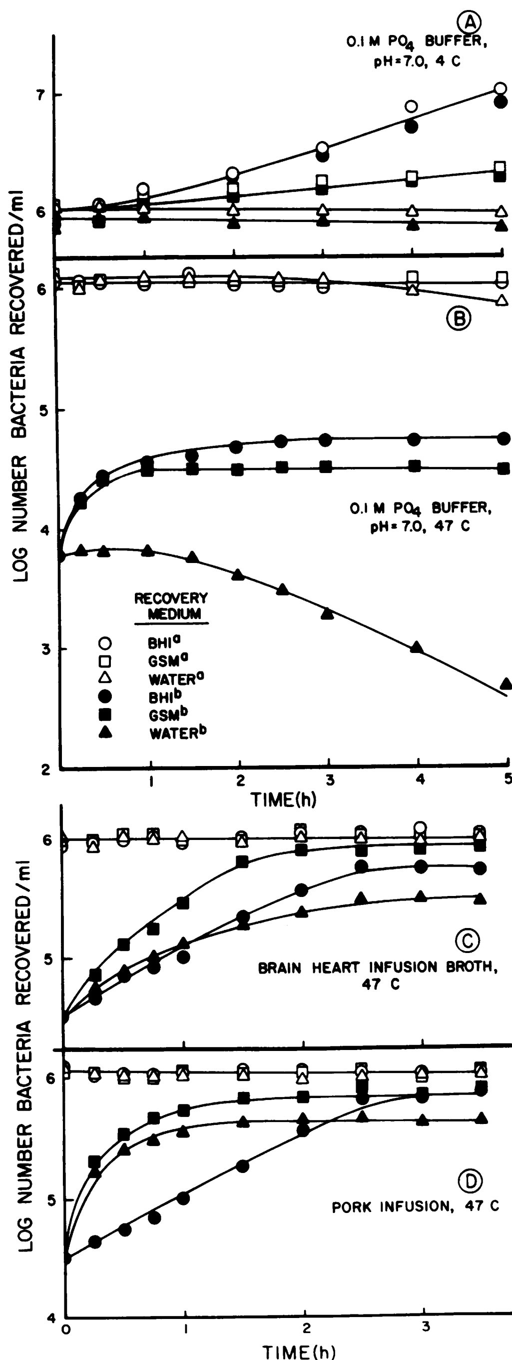

FIG. 4. *Recovery of heat-injured Y. enterocolitica serotype 0:17 cells in GSM, BHI broth, and water. Cells were heated in 0.1 M PO_4 buffer, pH 7.0, BHI broth, and PI for 12 min at 47°C. Controls used cells chilled for 12 min in the three menstruums. After heating and chilling, 10-ml volumes were centrifuged and the pellet was resuspended in sterile water. Two milliliters of washed cells was added to 98 ml of BHI broth, GSM, and sterile distilled water. a, BHI agar; b, TSA plus 0.16% BS.*

type 0:17 cells injured in the three heating menstruums required participation of RNA synthesis for their resuscitation in BHI broth.

The recovery curves in the presence of 2,4-dinitrophenol (155 μg/ml), an inhibitor of adenosine triphosphate (ATP) synthesis via oxidative phosphorylation in the electron transport system (8), are presented in Fig. 6. When *Y. enterocolitica* serotype 0:17 cells were heat injured in BHI or PI, the rate and extent of resuscitation in BHI broth were the same in the presence and absence of 2,4-dinitrophenol (Fig. 6B, C). However, for cells injured in PO_4 buffer, 2,4-dinitrophenol reduced the rate of repair (Fig. 6A). After 1 h, 0.63 and 0.43 log number of bacteria per ml of PO_4-injured cells resuscitated in the absence and presence of 2,4-dinitrophenol, respectively. With a further incubation (2 to 3 h), the amounts of resuscitation for these injured cells in the presence and absence of this metabolic inhibitor were equivalent. Therefore, cells

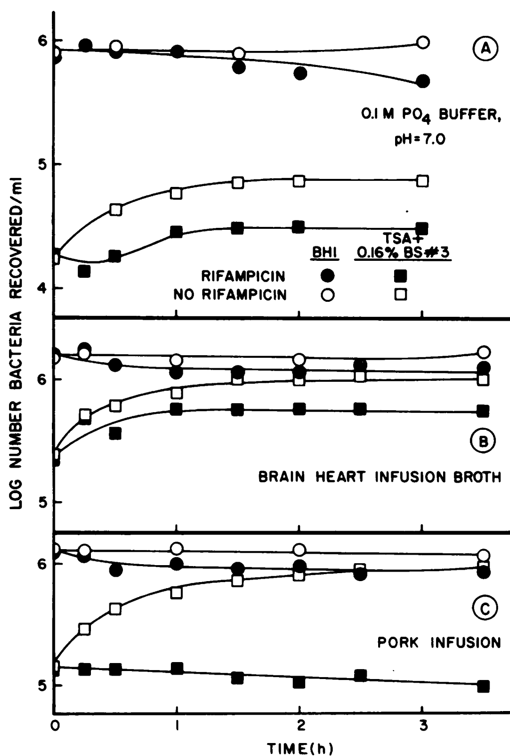

FIG. 5. *Effect of rifampin on the recovery of heat-injured Y. enterocolitica serotype 0:17 cells. Cells were heated in (A) 0.1 M PO_4 buffer, pH 7.0, (B) BHI broth, and (C) PI for 12 min at 47°C. After heating, injured cells were recovered at 25°C in BHI broth and BHI broth containing 8 μg of rifampin per ml.*

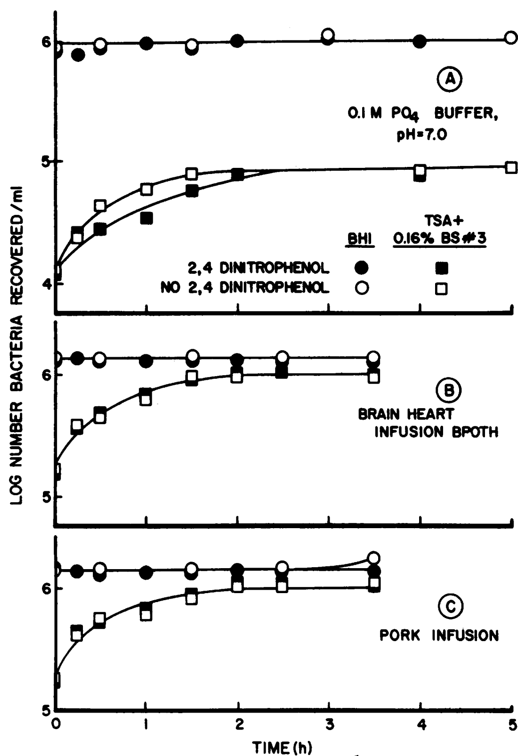

FIG. 6. *Effect of 2,4-dinitrophenol on the recovery of heat-injured Y. enterocolitica serotype 0:17 cells. Cells were heated in (A) 0.1 M PO_4 buffer, pH 7.0, (B) BHI broth, and (C) PI for 12 min at 47°C. After heating, injured cells were recovered at 25°C in BHI broth and BHI broth containing 155 µg of 2,4-dinitrophenol per ml.*

injured in PO_4 required energy via oxidative phosphorylation to repair at a maximal rate during the initial 1.5 h after incubation in BHI, whereas the energy from this source was not necessary to resuscitate at a normal rate for cells injured in BHI or PI.

DISCUSSION

An atypical strain, serotype 0:17, was more sensitive to BS and to sublethal heat treatment at 47°C in a PO_4 menstruum than the clinical strains, serotypes 0:3 and 0:8. Environmental (atypical) strains of *Y. enterocolitica* are more sensitive than the clinical (typical) isolates to environmental stresses such as heating (25), NaCl, pH (55), and certain selective compounds (9, 10) commonly used in isolation plating media for enteric microorganisms. The results of this investigation confirm these observations.

Hanna et al. (24) demonstrated that artificially contaminated frozen meat gave counts on bismuth sulfite plates that were consistently lower than counts on TSA plates. This implies that cells were sublethally injured during freezing. In our study, serotype 0:17 cells were stressed at 47°C in only 12 min. Therefore, subjecting a food product to mild heat treatment or freezing causes *Y. enterocolitica* cells to become stressed. Thus, enrichment procedures must be implemented in order to detect the injured cells.

The times required for thermally injured microorganisms to resuscitate in a recovery medium are mainly calculated from data obtained when cells are stressed in PO_4 buffer (14, 15, 18, 19, 31, 33, 35, 37, 57). Since PO_4 can chelate magnesium (R. S. Flowers, personal communication) resulting in the loss of cellular magnesium from the sublethally heated cells, this type of heating menstruum could create an artificial situation in which the mechanisms or extent of sublethal heat injury may be different than in a food menstruum. In this investigation, the length of the extended lag phase and resuscitation period of heat-injured *Y. enterocolitica* serotype 0:17 cells were dependent on the menstruums used. When these cells were heat injured at 47°C in 0.1 M PO_4 buffer for 12 min and subsequently inoculated in BHI broth tempered at 25°C, the recovery period for the heat-injured cells was 5 h, whereas cells heat injured in BHI or PI had a 2-h recovery period. McCoy and Ordal (44) demonstrated that *Pseudomonas fluorescens* cells grown, stressed, and recovered in complex medium had a greater resistance to heat and required a longer time to resuscitate than in a minimal medium system (22). Therefore, the times required for resuscitation of injured microbial cells in a recovery medium may be longer when heated in PO_4 versus a particular food environment. In other words, this extended recovery period of heat-injured cells in PO_4 may be longer than that required for recovery of cells injured in a food system. Since stressed pathogenic bacteria in foods may cause human disease similar to those caused by their unstressed counterparts (50), techniques to resuscitate them are extremely important. If the injured pathogenic microorganism that is being isolated is in low numbers in a particular food product, and if the incubation period is extended beyond the time needed for resuscitation, multiplication of nonpathogenic microorganisms could occur and suppress the pathogen, thus causing a decrease in the efficiency of isolation. Since *Y. enterocolitica* can grow at refrigerated temperatures (41), a cold enrichment procedure has been recommended for the isolation of this microorganism. However, this procedure has not been fully evaluated for recovery of injured *Y. enterocolitica* cells. Therefore, incubating the *Y. enterocolitica*

in primary isolation media at 25°C for 2 to 3 h before incubation at 4°C could produce an environment favorable for resuscitation and subsequent recovery of injured *Y. enterocolitica* cells. Thus, the efficiency of isolation would be increased.

When *Y. enterocolitica* 0:17 cells were sublethally heated in complex media (BHI broth or PI), the rate of recovery was faster in GSM than in a nutritionally complex medium such as BHI broth at 25°C. These results suggest that increased metabolic activity during recovery in a complex medium may retard the rate of resuscitation. Increased metabolic activity during resuscitation in nutritionally complex medium could result in an accumulation of H_2O_2 due to the destruction of catalase by heat (3, 13). *Y. enterocolitica* 0:17 cells injured in PI or BHI broth recovered in distilled water, whereas cells injured in 0.1 M PO_4 failed to recover, but rather sustained and died progressively after 1 h (Fig. 4). These results provide further evidence that sublethal heating in PO_4 buffer may cause different or more extensive destruction in heat-injured cells than in a complex medium. Since recent studies indicate that organisms resuscitate sublethal damage more readily in a simple minimal medium than in complex medium (33) (Fig. 4), testing individual and mixed components of the GSM may provide the minimal requirements for resuscitation of *Y. enterocolitica* cells heated in PO_4 buffer versus a complex medium.

Several sites of damage have been reported in thermally stressed microorganisms (15, 18, 21, 22, 30, 37, 44, 46, 57). Other manifestations of sublethal heat injury are alterations in nutritional requirements (1) and the dependence of ATP synthesis by oxidative phosphorylation (57). Data of others (18, 21, 22, 30, 37, 44) concerning the sites of cellular damage in thermally stressed microbial cells were derived from cells heated in PO_4 buffer. However, biosynthetic activities in one strain damaged under certain environmental conditions may be unique (40). In this investigation, the participation of RNA synthesis was necessary for normal resuscitation of injured *Y. enterocolitica* 0:17 cells, but the extent of this participation was dependent on the menstruum in which the cells were heated. When cells were heated in PO_4 buffer or BHI broth, partial resuscitation of the injured cells occurred in the presence of rifampin (RNA synthesis inhibitor), indicating that part of the injured cell population did not require RNA synthesis for recovery. Since rifampin inhibits only the initiation of RNA synthesis but not polymerization (58), a portion of the injured cell population heated in PO_4 buffer or BHI broth could have contained RNA precursors which would have resuscitated in the presence of the antibiotic. However, RNA synthesis was required for the resuscitation of all the injured *Y. enterocolitica* 0:17 cells heated in the PI menstruum (Fig. 5C). These data further suggest that even in complex heating menstruums, sites of cellular damage or the extent of damage at a particular cellular site could be different due to the physiocochemical composition of the menstruums. Therefore, since foods and food products vary widely in their chemical composition, the mechanism of thermal injury for a particular microorganism could be influenced by the food.

Inhibition of respiration by 2,4-dinitrophenol did not inhibit the recovery of *Y. enterocolitica* 0:17 cells heat injured in PI or BHI broth. However, for cells thermally injured in 0.1 M PO_4 buffer, this metabolic inhibitor slowed the rate but not the extent of resuscitation. The recovery medium (BHI broth) used in this investigation contains glucose as a carbon and energy source. Since oxidative phosphorylation was blocked, sufficient ATP production could occur through substrate-level phosphorylation to aid in the normal rate of recovery for *Y. enterocolitica* cells heated in BHI broth or PI, but not for cells heated in PO_4 buffer. These observations are in contrast to data which showed that 2,4-dinitrophenol was ineffective against *Vibrio parahaemolyticus* (30) and *Staphylococcus aureus* (37) heat stressed in 0.1 M PO_4 buffer and recovered in complex media containing glucose. Although lesions in the cellular membrane occur in injured *P. fluorescens* heated in a complex medium (S. E. Martin, personal communication), lesions induced by heating in a PO_4 buffer could be more massive and frequent due to the leaching of cellular magnesium into the menstruum (36). This would cause a depletion from the cell of this element which is needed for the stabilization of cellular membranes (56). Consequently, it could be possible that larger quantities of ATP molecules were able to leak from *Y. enterocolitica* 0:17 cells heated in PO_4 buffer, which could cause greater reduction in cellular ATP levels in these injured cells than in cells injured in BHI broth or PI. Alternatively, adenosine triphosphatase activity may have been greater in *Y. enterocolitica* 0:17 cells heat injured in PO_4 buffer than in cells injured in BHI broth or PI. Therefore, the production of ATP solely by substrate-level phosphorylation, which generates ATP molecules at a slower rate than oxidative phosphorylation (42), could be the rate-limiting factor during the early resuscitation of injured *Y. enterocolitica* 0:17 cells heated in PO_4 buffer.

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