Heat Injury and Repair in *Campylobacter jejuni*

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Received 23 April 1984/Accepted 11 June 1984

A procedure for detecting and quantitating heat injury in *Campylobacter jejuni* was developed. Washed cells of *C. jejuni* A7455 were heated in potassium phosphate buffer (0.1 M, pH 7.3) at 46°C. Samples were plated on brucella agar supplemented with $Na_2S_2O_3$, $FeSO_4 \cdot 7H_2O$, and sodium pyruvate and on a medium containing brilliant green, bile, $Na_2S_2O_3$, $FeSO_4 \cdot 7H_2O$, and sodium pyruvate. Colonies were counted after 5 days of incubation at 37°C in an atmosphere containing 5% O_2, 10% CO_2, and 85% N_2. After 45 min at 46°C, there was virtually no killing and ca. two log cycles of injury. Cells grown at 42°C were more susceptible to injury than cells grown at 37°C. The addition to brucella agar supplemented with $Na_2S_2O_3$, $FeSO_4 \cdot 7H_2O$, and sodium pyruvate of three different antibiotic mixtures used in the isolation of *C. jejuni* from foods or clinical specimens did not prevent recovery of heat-injured *C. jejuni*. Cells lost 260 nm of absorbing materials during heat injury. The addition of 5% NaCl or 40% sucrose to the heating buffer prevented leakage but did not prevent injury. Of the additional salts, sugars, and amino acids tested for protection, only NH_4Cl, KCl, and $LiCl_2$ prevented injury. Heat-injured *C. jejuni* repaired (regained dye and bile tolerance) in brucella broth supplemented with $Na_2S_2O_3$, $FeSO_4 \cdot 7H_2O$, and sodium pyruvate within 4 h. Increasing the NaCl in this medium to 1.25% inhibited repair, and increasing it to 2% was lethal. Heat-injured *C. jejuni* will repair at 42°C but not at 5°C.

Campylobacter jejuni is emerging as a bacterium of considerable importance as an agent of human diarrhea (1, 4). Some reports have even indicated that *C. jejuni* may be isolated from human diarrhea cases as frequently as *Salmonella* spp. (1, 8). The mode of transmission is currently unknown, although food has been suggested as a vehicle (2, 4). *C. jejuni* has been isolated from poultry (9, 12) as well as from various other foods, such as raw milk, beef, and pork (2, 4, 6, 19).

Many foodborne microorganisms, including pathogens, may be stressed (sublethally injured) by food processing unit operations such as heating, freezing, drying, fermentation, and acidification (3, 18). Heat injury has been demonstrated in organisms such as *Staphylococcus aureus*, salmonellae, streptococci, pseudomonads, yeasts, molds, and spores (3, 18). It became of interest to determine whether the phenomenon of heat injury could be demonstrated in *C. jejuni* and to assess whether some procedures currently used for the isolation of *C. jejuni* from foods would detect heat-injured *C. jejuni*.

MATERIALS AND METHODS

Organism and preparation of culture. *C. jejuni* A7455, obtained from L. Blankenship, was used throughout these studies. The organism was grown for 24 h at 37°C in brucella broth (Difco Laboratories, Detroit, Mich.) supplemented with BFP (sodium metabisulfite, ferrous sulfate, and sodium pyruvate [each at 0.25 g/liter] [7]) (BBS). The atmosphere above the liquid (125 ml of BBS in a 500-ml Erlenmeyer flask) was evacuated and then filled with a gas mixture containing 5% O_2, 10% CO_2, and 85% N_2 (OCN). The growing culture was agitated on a reciprocating shaker (model G 33; New Brunswick Scientific Co., Inc., Edison, N.J.) at 175 rpm. After the 24-h incubation period (early stationary phase), the cells were harvested by centrifugation (16,300 × *g*, 5°C), washed three times with sterile potassium phosphate buffer (0.1 M, pH 7.3), and resuspended in a small quantity of sterile buffer.

Injury. Sterile potassium phosphate buffer (0.1 M, pH 7.3) was heated to 46°C with stirring. After that temperature was attained, the *C. jejuni* suspension was added, and timing was started. About 3 min was required for the buffer to regain that temperature after addition of the cells. At intervals, aliquots were removed, and appropriate dilutions were made in 0.1% peptone (Difco) water and surface plated on brucella agar supplemented with BFP (BAS) and on BGBS (brilliant green–2% bile broth [Difco], 0.5% NaCl, 2% agar, and BFP). Plating was done on a Spiral plater (model DU; Spiral Systems, Bethesda, Md.). Plates were incubated for 5 days at 37°C in the OCN atmosphere and then counted.

Leakage and protection. To determine whether *C. jejuni* lost any cellular components as a result of heat injury, I removed aliquots during the injury sequence, centrifuged them (12,100 × *g*, 5°C), and scanned the cell-free buffers (200 to 400 nm, 1-cm quartz cuvette) in a Hewlett-Packard model 8450 A UV/VIS spectrophotometer against fresh sterile buffer.

Studies with other bacteria have determined that the addition of various solutes to the heating buffer protects these organisms from the deleterious effects of heat (16, 17). This was investigated in *C. jejuni* by adding various sugars, salts, and amino acids to the heating buffer. Aliquots of the culture were plated as described above.

Repair. As a result of injury, *C. jejuni* lost dye and bile tolerance. To investigate repair (regaining of dye and bile tolerance), I centrifuged heat-injured *C. jejuni* (12,100 × *g*, 5°C), suspended it in fresh BBS, and gassed it with OCN. The flask was incubated at 37°C with shaking, and at intervals, aliquots were removed and plated on BAS and BGBS as described above. These plates were incubated at 37°C in OCN for 5 days and then counted.

RESULTS AND DISCUSSION

Assay procedure. In the development of a procedure to detect and quantitate injured *C. jejuni* cells, several factors had to be considered. From preliminary experiments in

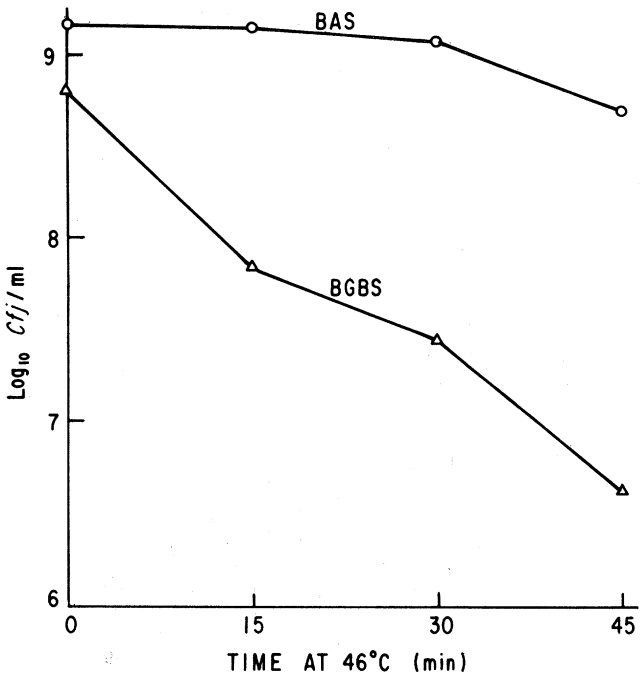

FIG. 1. Effect of plating medium on the recovery of *C. jejuni* (*Cfj*) heated in phosphate buffer (0.1 M, pH 7.3) at 46°C. (See text for compositions of BAS and BGBS.)

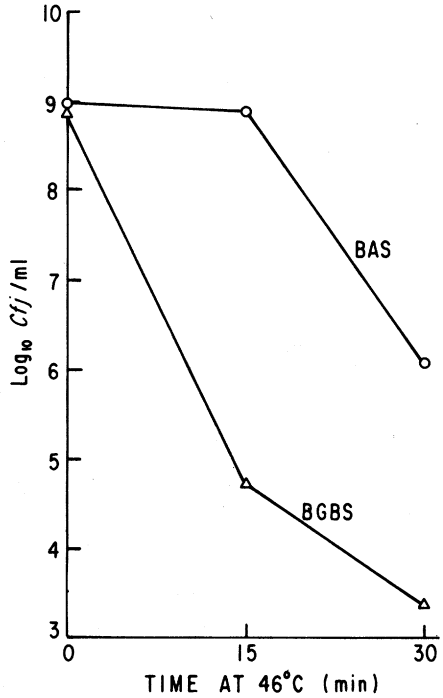

FIG. 2. Injury of *C. jejuni* (*Cfj*) grown at 42°C. (See text for compositions of BAS and BGBS.)

which washed cell suspensions of *C. jejuni* were heated in phosphate buffer (0.1 M, pH 7.3) at temperatures of 44 to 48°C, it was determined that heating at 46°C for periods of up to 45 min yielded no change or very few changes in viable counts after plating on BAS. Thus, 46°C was chosen as the heating temperature, and BAS was chosen as the control medium, and all counts were compared with the count on BAS. Next, a differential medium, one on which counts would decrease with heating time, was sought, the difference between BAS and the differential medium representing the number of injured cells. After evaluation of a number of potential selective agents, it was found that a medium containing brilliant green and bile (oxgall) (BGBS) filled this requirement. Data from a typical experiment are shown in Fig. 1. Inclusion of the BFP supplement was particularly critical in that, as a gram-negative rod, *C. jejuni* should grow on dye-containing media such as MacConkey agar (13), eosin methylene blue agar, and violet-red bile agar. However, without the BFP supplement, there was a much lower recovery of unheated cells on these media.

The addition of brilliant green (final concentration, 0.0133 g/liter, the same concentration as in BGBS) to BAS produced a very inhibitory medium, one on which even unheated cells would not grow. Increasing the NaCl level to 1% also produced an inhibitory medium. Inclusion of 1% glycine, 0.1% sodium acid selenite, 1% bile salts no. 3, 0.001 M iodoacetate (13, 14), or triphenyltetrazolium chloride (final level, 400 μg/ml) (11) also proved unsatisfactory.

A five-day incubation of BAS and especially BGBS plates is also critical. Less incubation of BGBS plates yielded fewer and smaller colonies.

C. jejuni cells grown at 42°C were also subjected to the injury procedure described above (Fig. 2). Growth at this elevated temperature yielded cells which were more readily injured than cells grown at 37°C. In addition to being more readily injured, these cells also died more rapidly than cells grown at 37°C (cf. the 30-min counts on BAS and BGBS in Fig. 1 and 2).

Antibiotics-supplemented media. Antibiotics are often added to media used to isolate *C. jejuni* from clinical specimens and foods (6). Although certain antibiotics do not interfere with the growth of healthy, normal cells, it is known that they can interfere with the recovery of heat-injured *S. aureus* (10). Three of the antibiotic mixtures commonly used to isolate *C. jejuni* from foods and clinical specimens were added to BAS, and heated *C. jejuni* cells were plated (Table 1). Counts on the three antibiotics-supplemented media during heating indicated that none of the antibiotics interfered with the growth or recovery of heat-injured *C. jejuni*.

Leakage and protection. Because of damage to the cell membrane caused by heating, cells often lose cellular com-

TABLE 1. Influence of the addition of antibiotics to BAS on the recovery of *C. jejuni* heated in phosphate buffer at 46°C

Plating medium	Log_{10} no. of *C. jejuni* cells at indicated heating time (min)		
	0	20	40
BAS[a]	9.40	9.18	7.90
BGBS[a]	8.96	7.75	5.78
BAS + Butzler[b]	8.28	8.11	7.69
BAS + Skirrow[c]	9.30	9.18	8.00
BAS + Blaser-Wang[d]	9.30	9.18	7.93

[a] See text for compositions of BAS and BGBS.

[b] Butzler antibiotic mixture (Oxoid SR-85; Oxoid Ltd., Basingstoke, England) contained the following: cycloheximide, 0.05 mg/ml; bacitracin, 25U/ml; colistin sulfate, 10 U/ml; cefazolin sodium, 0.015 mg/ml; and novobiocin, 0.005 mg/ml.

[c] Skirrow antibiotic mixture (Oxoid SR-69; Oxoid) contained the following: vancomycin, 0.01 mg/ml; trimethoprim lactate, 0.005 mg/ml; and polymyxin B, 2.5 U/ml.

[d] Blaser-Wang antibiotic mixture (Oxoid SR-98; Oxoid) contained the following: vancomycin, 0.01 mg/ml; polymyxin B, 2.5 U/ml; trimethoprim, 0.05 mg/ml; amphotericin B, 0.002 mg/ml; and cephalothin, 0.015 mg/ml.

ponents during injury. *C. jejuni* is typical in that materials absorbing at 260 nm leak into the heating buffer during injury. This would indicate both damage to the cell membrane during heat injury and some degradation of cellular components. Studies on *S. aureus* (17) have indicated that the addition of various solutes, such as salts, sugars, and amino acids, to the heating buffer can negate or circumvent the deleterious effect of heat. This was investigated in *C. jejuni*. It was found that the addition of 5% NaCl or 40% sucrose to the heating buffer prevented the leakage of materials absorbing at 260 nm into the heating buffer but did not prevent injury to the cell.

Using methods developed by Smith et al. (16), I tested other solutes and evaluated the data to determine if they could prevent heat injury to *C. jejuni* (Table 2). The salts tested presented two extremes: two ($MgCl_2$ and NaCl) gave no protection, and the other three gave relatively good protection. The three sugars offered no protection, and of the two amino acids tested, one offered no protection, and one offered virtually no protection. These data for heat-injured *C. jejuni* are in contrast to data for protection in heat-injured *S. aureus* in that almost all solutes tested gave substantial protection to *S. aureus* (16, 17). Whether this represents differences between gram-negative and gram-positive bacteria or differences in the mechanism(s) of heat damage in the two organisms is not known. The exact mechanism by which these solutes prevent heat injury in *S. aureus* is not known (15–17).

Repair. One characteristic of injured as opposed to dead cells in their ability to repair the cellular damage caused by heat. As both injured and noninjured cells formed colonies on BAS, BBS was chosen for study as a possible repair medium. After injury, the cell suspension was centrifuged, suspended in fresh BBS, gassed with OCN, and incubated at 37°C with agitation. At intervals, aliquots were plated on BAS and BGBS (Fig. 3a). Repair was complete by 4 h of incubation.

As many foods are refrigerated after heat processing, it was of interest to determine if heat-injured *C. jejuni* could repair at low temperatures. After being heated, *C. jejuni* was processed as described above except that the incubation temperature was 5°C. Aliquots were plated daily. There was no repair at 5°C; in fact, there was a gradual decline in both BAS and BGBS counts over the 5-day period.

Physiologically, *C. jejuni* is characterized by its ability to grow at 42°C (13, 14). Doyle and Roman (6) took advantage of this characteristic by incorporating a 42°C enrichment stem into their procedure for recovery of *C. jejuni* from contaminated foods. Thus, it was of interest to determine whether heat-injured *C. jejuni* could repair in BBS held at 42°C (data not shown). No further injury occurred after incubation at 42°C, and repair (regaining of dye and bile tolerance) occurred within 4 h. Thus, a 42°C enrichment step would not be contraindicated for use in isolating *C. jejuni* from heat-processed foods.

TABLE 2. Effect of solutes in the heating buffer on the response of *C. jejuni* to heat injury

Compound	Relative protective effect[a]
5% NaCl	0
$MgCl_2$[b]	0
NH_4Cl[b]	0.99
KCl[b]	0.77
$LiCl_2$[b]	0.94
40% Sucrose	0
25% Sorbitol	0
25% Glycerol	0
10% β-Alanine	0.09
10% Glycine	0
Skim milk (10% nonfat dry milk in distilled water)	0.38

[a] A relative protective effect of 1 = complete protection, with counts equal to those on BAS; a relative protective effect of 0 = no protection, with counts less than or equal to those on BGBS.

[b] Molar equivalent to 5% NaCl, 0.85 M.

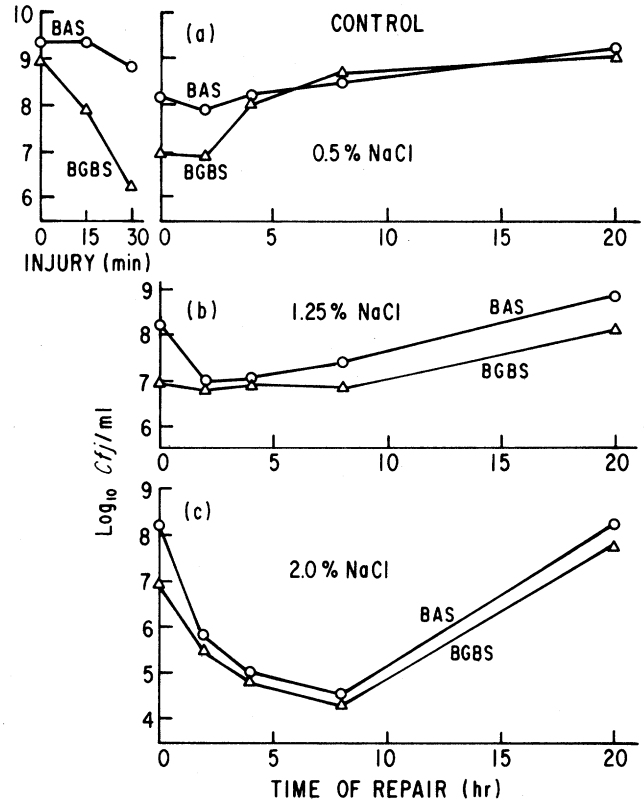

FIG. 3. Effect of NaCl level on repair of heat-injured *C. jejuni* (*Cfj*). (See test for compositions of BAS and BGBS.) (a) Control (0.5% NaCl, the amount present in BBS). (b) 1.25% total NaCl in BBS. (c) 2.0% total NaCl in BBS.

Many food products, and especially processed meat and poultry products, contain various levels of NaCl. The effect of different levels of NaCl on repair of heat-injured *C. jejuni* was studied by adding NaCl to BBS (Fig. 3). The injury data for these cells are shown to the left of Fig. 3a. Fig. 3a represents the control, 0.5% NaCl, the amount present in BBS. As indicated above, repair occurred by 4 h of incubation. Increasing the levels above the basal 0.5% had an adverse effect on repair. A 2% total NaCl concentration (Fig. 3c) caused a decrease in the viable counts (BAS and BGBS) up to 8 h, when a regrowth of the culture occurred. At the intermediate level (1.25% total NaCl concentration) (Fig. 3b), there was a decrease in the BAS counts at 2 h, until they were the same as the BGBS counts. With further incubation, the BAS counts increased, and the BGBS counts also increased, although to a lesser extent. This suggested that this level of salt (1.25%) inhibited repair. Work on the development of a differential plating medium for the detection of heat-injured *C. jejuni* indicated that a 1.5% total NaCl concentration in BAS allowed a much smaller number of *C.*

jejuni cells to form colonies after heating, thus supporting the observations on repair in BBS.

The above observations on the NaCl sensitivity of *C. jejuni* are supported by the observations of Doyle and Roman (5). They found that *C. jejuni* grew best at 0.5% NaCl but indicated that concentrations as low as 1% may retard growth of the organism and are contraindicated for use in media used to recover or enumerate this organism. Further, repair of heat injury in *C. jejuni* appears to be as salt sensitive as growth.

This work indicates that *C. jejuni* can be injured at 46°C and that this injury can be detected by use of a plating medium containing brilliant green and bile (oxgall). The data presented indicate that the three commonly used antibiotic mixtures do not interfere with the recovery of heat-injured *C. jejuni*. In addition to the observation that heat-injured *C. jejuni* would repair, it was also observed that repair occurred at 42°C. This last observation indicated that a 42°C enrichment step or a 42°C incubation of plates would not be contraindicated for isolating *C. jejuni* from heat-processed foods. The salt sensitivity of the organism was verified at levels of 1.25 to 1.5% during both repair studies and development of a plating medium.

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