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Relationships of Temperature and Sodium Chloride Concentration to the Survival of *Vibrio parahaemolyticus* in Broth and Fish Homogenate[1]

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The interaction of temperature and NaCl concentration in affecting the survival of three strains of *Vibrio parahaemolyticus* was studied in Trypticase soy broth and fish homogenate. Cells of *V. parahaemolyticus* suspended in Trypticase soy broth without NaCl were quite unstable and readily killed. The presence of NaCl appeared to be protective to the cells at 48 ± 1 C, with the optimal concentration strain-dependent for the 3 to 12% range tested. Temperatures of 5 ± 1, −5 ± 1, and −18 ± 1 C reduced the number of viable organisms per milliliter regardless of the NaCl concentration. In the presence of NaCl, viable cells, in numbers ranging up to 580 per ml, were still detected at the end of 30 days of storage. Similar results were obtained for cells suspended in fish homogenate, except that fish homogenate itself was protective as compared with Trypticase soy broth. This protection was significantly lower than that provided by NaCl in any amount tested.

The survival of *Vibrio parahaemolyticus* in foods is of concern since this organism is a major cause of gastroenteritis in Japan, and four outbreaks (3, 9) have possibly been identified in the United States. *V. parahaemolyticus* has been isolated from the coastal waters and fish of the United States; thus, the potential exists for it to be a cause of a greater number of outbreaks here.

Many of the peccant foods in Japanese outbreaks have been sea fish or salted vegetables. Since *V. parahaemolyticus* is halophilic, optimal concentrations of salt may increase cellular resistance to stress conditions. Temmyo (10), who studied the prevention of food-poisoning outbreaks caused by this organism in Japan, concluded that 5 and 7% NaCl could enhance survival in saurel extracts during storage at −18 C for short periods of time (4 days). Asakawa (1) reported a much greater rate of decline in numbers of *V. parahaemolyticus* cells in laboratory media at −10 C than at −20 C. Survival was greatest at 0 C. He also inoculated raw tuna meat and found survival was about the same at −20 C as at −10 C and that survival was greater at both than at 0 C. Based on studies on the lethal effects of freezing at −10 and −24 C and heating at 60 C in phosphate buffer and fish homogenate, Liston and his associates concluded that *V. parahaemolyticus* appeared to be more readily inactivated by freezing and heating than other food-poisoning organisms (7). The organism tended to die rapidly when held below 5 C (5). There was greater survival of the organism at 0.6 C than at lower temperatures in cooked-fish homogenate (8). However, survival occurred under retail conditions, since the organism was repeatedly isolated from commercial shellfish samples (6).

The present study was initiated to provide information concerning the survival of *V. parahaemolyticus* in fish homogenate and in Trypticase soy broth (TSB) with various NaCl concentrations during heating and low-temperature storage.

MATERIALS AND METHODS

Cultures. Three strains of *V. parahaemolyticus* were used: T-3765—1 (from H. Zen-Yoji, Tokyo-to

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Laboratories for Medical Sciences, Tokyo, Japan, isolated from a Japanese food-poisoning patient), SB04-422 (from J. Baross, University of Washington, Seattle, isolated from oysters in the study of the Seabeck outbreak), and ATCC 17802 (originally isolated from a Japanese food-poisoning patient). Cultures were maintained on 3% NaCl-Trypticase soy agar (TSA, BBL) slants at room temperature. For inocula, cultures were grown for 48 hr on the same medium at 25 C. Slants were washed with each of the NaCl-TSB combinations. Dilutions were made with broth of the same concentration to give a final population of 10,000 cells per ml. The five NaCl concentrations were included in random order each day.

Systems. TSB (4) was used as a basic system with three replications for each strain-temperature combination. A frozen and thawed fish homogenate, composed of sturgeon, ling cod, and black rockfish prepared to have a minimal number of contaminating microorganisms, was used as a food system with two replications. The fish homogenate was prepared by diluting the fish muscle with nine parts of NaCl solution and blending for 3 min at low speed in an electric blender after inoculation. NaCl was added to both systems at concentrations of 0, 3, 6, 9, and 12%.

Recovery media. Preliminary experimentation showed no appreciable differences in the recovery of stressed cells in nutrient agar (BBL) with 3% NaCl, TSA with 3% NaCl, and Liston's maintenance medium (2). TSA containing 3% NaCl was chosen for recovery of cells in all experiments. For recovery of cells heated in 6% NaCl medium, both 3 and 6% levels of NaCl were used in the TSA. Data are reported only for 3%, which gave higher recovery. Diluent was 0.1% peptone-water with the corresponding NaCl concentration. Plates were incubated for 24 hr at 37 C.

Procedure. Temperatures compared were 48 ± 1 C in a water bath, 5 ± 1 C in a water bath, −5 ± 1 C in a water-ethylene glycol bath, and −18 ± 1 C in air in a freezer. Ampoules (5-ml) containing the sample were used, with one ampoule removed at each sampling period. Sampling times at 48 ± 1 C were 0.5, 5, 10, 20, and 40 min plus an 80-min sample for fish homogenate. Time required to reach this temperature in TSB, as determined by means of a recording pyrometer with thermocouples, was 1.3 min; in fish homogenate, the time was 4 min. Storage periods for the low-temperature studies were 1, 9, 16, and 30 days. Time before the low temperature was reached in TSB at 5 ± 1 C was 2.7 min and at −5 ± 1 C, 3.3 min. At −18 ± 1 C, there was a rapid decrease to below 0 C in 10 min with slower cooling through about 70 min. In fish homogenate at −5 ± 1 C, lag time ranged from 3.6 min in 0% NaCl to 4.7 min for 6, 9, and 12% NaCl. At −18 ± 1 C, a cooling pattern similar to that of TSB was observed.

A factorial design was used for the experiment for the two systems at 48 ± 1 C and for the low temperature study. Logarithmic transformations of the data were done for the statistical analysis. Significant effects (0.05 level or greater) are reported. Least significant differences were used for comparisons between NaCl concentrations.

RESULTS

TSB. In TSB heated at 48 ± 1 C, the presence of NaCl in any concentration tested resulted in significantly greater recovery of viable cells of all three strains than occurred in its absence. Data are shown by the broken lines in Fig. 1 for strain 17802, and in Table 1 for strain SB04-422. Data for T-3765-1 were similar except for greater survival in fish homogenate without added NaCl. Level of NaCl and sampling time were each significant for all strains, as was the interaction of the two for two strains.

Storage at all low temperatures in TSB resulted in reduced numbers of viable organisms, but in the presence of NaCl numbers ranging up to 580 per ml were still detected at the end of the 30-day storage period. However, very low numbers (<30 per ml) were generally recovered.

Significant differences appeared for only one strain in comparing the three low temperatures. For strain T-3765-1, survival at −5 ± 1 C was significantly less than at 5 ± 1 and −18 ± 1 C. Effect of sampling times and NaCl concentrations were significant for all three strains. NaCl concentrations of 0 and 3% resulted in significantly lower counts of survivors than other levels for strains T-3765-1 and SB04-422. Data in Table 2 illustrate the differences between 0 and 6% NaCl-TSB for strain SB04-422 at three temperatures. For the other two strains, Fig. 2 presents a comparison of the effect of NaCl concentration at −18 ± 1 C. For strain 17802, broths with 0% NaCl gave significantly lower recoveries than all other concentrations.

Fish homogenate. Fish homogenate without additional NaCl tended to stabilize the cells of *V. parahaemolyticus* at all temperatures when compared with 0% NaCl-TSB (Fig. 1 and Tables 1–3). However, added NaCl gave significantly increased numbers surviving at 48 ± 1 C for all strains. Data are presented for two strains (Table 1 and Fig. 1) as representative of the trends found.

Storage of the samples at −5 ± 1 and −18 ± 1 C, even in the presence of additional NaCl, resulted in considerable decreases in viable counts of *V. parahaemolyticus* (Table 3). The trends were similar for all three strains although the slopes varied. Sampling time and level of NaCl were each significant for all strains. Growth of contaminating gram-positive cocci occurred in the fish homogenate at 5

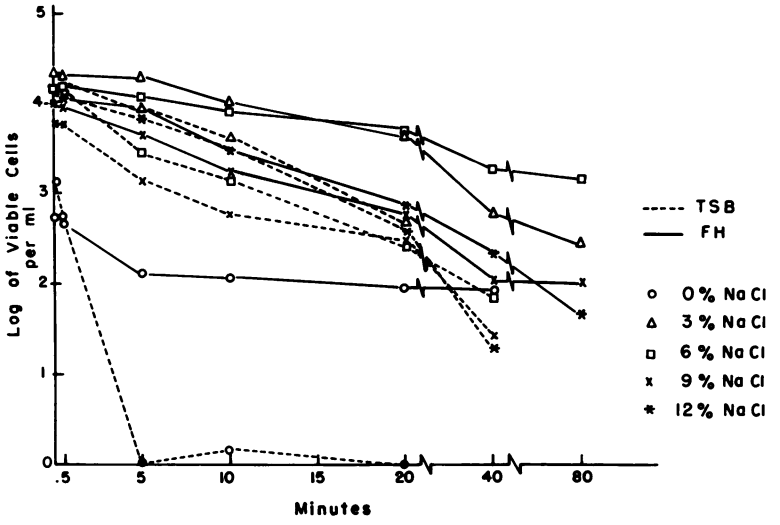

FIG. 1. *Survival of V. parahaemolyticus strain 17802 at 48 ± 1 C in Trypticase soy broth (TSB) and fish homogenate (FH) with various levels of NaCl (lag times to reach temperature, 1.3 min for broth and 4 min for fish homogenate).*

TABLE 1. *Numbers[a] of viable V. parahaemolyticus cells per milliliter for strain SB04-422 in Trypticase soy broth and fish homogenate with various levels of NaCl held at 48 ± 1 C for different time periods*

Sampling time (min)[b]	Menstruum	NaCl				
		0%	3%	6%	9%	12%
0	Trypticase soy	23×10^2	21×10^3	16×10^3	14×10^3	10×10^3
0.5		20×10^1	19×10^3	14×10^3	13×10^3	10×10^3
5		40×10^1	14×10^3	12×10^3	77×10^2	61×10^2
10		89	80×10^2	97×10^2	50×10^2	40×10^2
20		<30	41×10^2	72×10^2	38×10^2	13×10^2
40		—[c]	—[c]	33×10^2	25×10^2	84
0	Fish homogenate	50×10^1	22×10^3	16×10^3	16×10^3	10×10^3
0.5		44×10^1	20×10^3	16×10^3	12×10^3	12×10^3
5		76	22×10^3	14×10^3	12×10^3	74×10^2
10		53	16×10^3	14×10^3	11×10^3	52×10^2
20		40	15×10^3	14×10^3	94×10^2	22×10^2
40		<30	56×10^2	91×10^2	42×10^2	17×10^1
80		<30	40×10^2	55×10^2	16×10^2	<30

[a] Average of three replications for Trypticase soy and two for fish homogenate.
[b] Lag times to reach temperature were 1.3 min for broth and 4 min for fish homogenate.
[c] Not tested.

TABLE 2. *Numbers[a] of viable V. parahaemolyticus cells per milliliter for strain SB04-422 in Trypticase soy broth with 0 and 6% NaCl held at 5 ± 1, −5 ± 1 and −18 ± 1 C for different time periods*

Storage time (days)	5 ± 1 C		−5 ± 1 C		−18 ± 1 C	
	0% NaCl	6% NaCl	0% NaCl	6% NaCl	0% NaCl	6% NaCl
0	23×10^2	16×10^3	23×10^2	16×10^3	23×10^2	16×10^3
1	12×10^2	14×10^3	47	11×10^3	15×10^1	93×10^2
9	<30	14×10^2	<30	51×10^1	<30	19×10^2
16	31	81	<30	47×10^1	<30	49×10^1
30	<30	<30	<30	<30	<30	75

[a] Average of three replications.

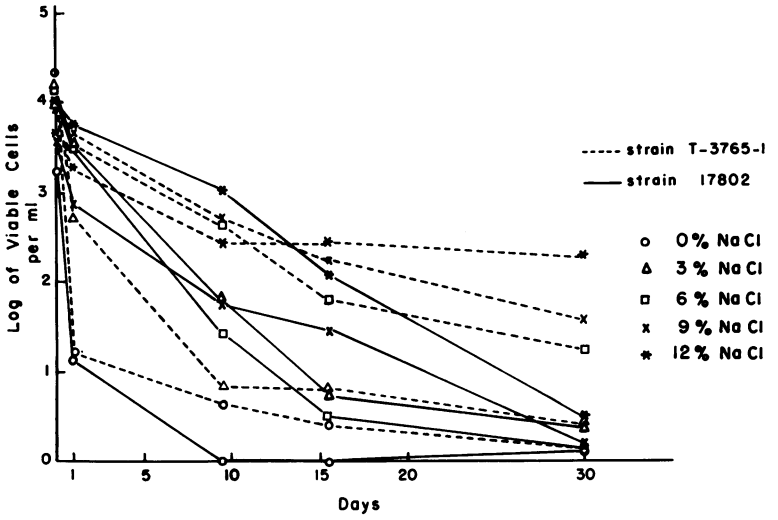

FIG. 2. *Survival of V. parahaemolyticus strains T-3765-1 and 17802 at −18 ± 1 C in Trypticase soy broth with various levels of NaCl.*

TABLE 3. *Numbers[a] of viable V. parahaemolyticus cells per milliliter for strains SB04-422 and T-3765-1 in fish homogenate with various levels of NaCl held at −5 ± 1 and −18 ± 1 C for different time periods*

Strain	Storage time (days)	0% NaCl		3% NaCl		6% NaCl		9% NaCl		12% NaCl	
		−5±1 C	−18±1 C	−5±1 C	−18±1 C	−5±1 C	−18±1 C	−5±1 C	−18±1 C	−5±1 C	−18±1 C
SB04-422	0	50×10^1	50×10^1	22×10^3	22×10^3	16×10^3	16×10^3	16×10^3	16×10^3	10×10^3	10×10^3
	1	86	32	68×10^2	10×10^3	13×10^3	92×10^2	12×10^3	12×10^3	80×10^2	75×10^2
	9	52	<30	71	12×10^1	15×10^1	19×10^2	15×10^1	15×10^2	<30	79×10^1
	16	40	<30	66	79	64	12×10^1	71	40	<30	77
	30	33	<30	39	46	<30	45	40	<30	<30	<30
T-3765-1	0	13×10^2	13×10^2	98×10^2	98×10^2	12×10^3	12×10^3	81×10^2	81×10^2	60×10^2	60×10^2
	1	50×10^1	28×10^1	23×10^1	58×10^2	40×10^2	10×10^3	34×10^2	42×10^2	44×10^2	40×10^2
	9	10×10^1	23×10^1	14×10^1	20×10^1	49	13×10^2	76	54×10^1	24×10^1	12×10^2
	16	45	17×10^1	52	<30	<30	50	<30	<30	89	<30
	30	<30	15×10^1	46	<30	78	40	<30	<30	48	78

[a] Average of two replications.

± 1 C and resulted in discarding of data at this temperature.

DISCUSSION

A relatively low inoculum level of 10^4 was used because this is in the range of numbers of vibrios recovered from shellfish samples (2). Differences among the three strains appeared, as was also observed by Matches et al. (8) and Temmyo (10). The very sharp drop in survivors if NaCl was not added to the medium was noted throughout the experiments. The organism was rapidly killed owing to effects other than heating or chilling. This resulted in often rather erratic results. It was very difficult to maintain the viable count even at room temperature. The zero-time counts and survival after 0.5 min of heating illustrate this effect. However, except for a significant difference among replications for strain 17802 in the low-temperature fish homogenate study, agreement among replications was good.

Addition of NaCl to the medium in which *V. parahaemolyticus* was heated or stored appeared to confer a stabilizing effect on the organism. Temmyo (10) found that NaCl in concentrations ranging from 0.5 to 7% appeared to give some protection to *V. parahaemolyticus* in saurel extracts stored for 8 days at −2 C and 6 days at −18 C. However, this stabilizing effect was insufficient to protect the cells for long-term storage at low temperatures.

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