APPLIED MICROBIOLOGY, June 1974, p. 1075-1080

Vol. 27, No. 6
Printed in U.S.A.

Combined Effects of Water Activity, Solute, and Temperature on the Growth of *Vibrio parahaemolyticus*

L. R. BEUCHAT

Department of Food Science, University of Georgia Agricultural Experiment Station, Experiment, Georgia 30212

Received for publication 25 February 1974

Vibrio parahaemolyticus was grown at 36 C in tryptic soy broth (pH 7.8) containing added levels of NaCl ranging from 0.5 to 7.9% (wt/wt). The fastest generation time was 16.4 min in tryptic soy broth containing 2.9% NaCl (TSBS) which corresponded to a water activity (a_w) of 0.992 ($\pm$0.005). Tryptic soy broth containing lower or higher levels of NaCl resulted in higher or lower a_w, respectively, and slower generation times. Growth was measured turbidimetrically at 36 C in TSBS containing added amounts of NaCl, KCl, glucose, sucrose, glycerol, or propylene glycol. The solutes used to reduce a_w to comparable levels resulted in extended lag times of varied magnitude, dissimilar growth rates, and different cell numbers. Reduction of a_w with glycerol was less inhibitory to growth than similar a_w reductions with NaCl and KCl. Sucrose, glucose, and propylene glycol generally had the greatest effect on extending the lag times of *V. parahaemolyticus* when the addition of these solutes was made to establish similar a_w levels lower than 0.992. Minimal a_w for growth at 15, 21, 29, and 36 $\pm$ 0.2 C for each of four strains of *V. parahaemolyticus* was tested in TSBS containing added solutes. Reduced a_w was generally most tolerable at 29 C, whereas higher minimal a_w for growth was required at 15 C. Solutes added to TSBS to achieve reduction in a_w, minimal a_w for growth after 20 days, and incubation temperatures were as follows: glycerol, 0.937, 29 C; KCl, 0.945, 29 C; NaCl, 0.948, 29 C; sucrose, 0.957, 29 and 36 C; glucose, 0.983, 21 C; and propylene glycol, 0.986, 29 C. Each of the four strains tested responded similarly to investigative conditions. It appears that minimal a_w for growth of *V. parahaemolyticus* depends upon the solute used to control a_w.

The relationships between available water content and the potential for spoilage of foods by microorganisms have been of interest for many years. Various minimal, optimal, and maximal moisture levels, usually expressed in terms of water activity (a_w), have been reviewed (17, 20). Much of the available data on a_w requirements for bacteria have resulted from studies on foodborne pathogens such as salmonellae (3, 4, 6), staphylococci (5, 15, 16, 19), and *Clostridium* spp. (1, 7, 10, 18). In light of the recent recognition of *Vibrio parahaemolyticus* as a cause of foodborne disease outbreaks in the United States (2) and the lack of definitive information regarding the organism's tolerance to reduced a_w levels, several experiments were designed to determine the growth response of *V. parahaemolyticus* over a wide range of a_w.

In the present study, the optimal a_w in a medium containing added NaCl was established. Effects from minor changes in optimal a_w levels resulting from the addition of several solutes to growth media on lag times of *V. parahaemolyticus* are discussed. Minimal a_w levels for growth at 15, 21, 29, and 36 C were achieved by the addition of solutes to a basal medium.

MATERIALS AND METHODS

Test cultures. Four strains of *V. parahaemolyticus* were studied: M5250J-2 (O1:K22), received from W. E. DeWitt, Center for Disease Control, Atlanta; 4750 (O2:K3) and T-3765-1 (O3:K7), obtained from H. Zen-Yoji, Tokyo Metropolitan Research Laboratory of Public Health; and 8700 (O4:K11), acquired from M. Fishbein, Food and Drug Administration, Washington, D.C. Stock cultures were maintained at room temperature in a long-term preservation medium consisting of 0.3% yeast extract, 1.0% peptone (Difco), 2.0% NaCl, and 0.3% agar in distilled water as suggested by M. Fishbein.

Media. The basal medium used for all investigations was tryptic soy broth (TSB, Difco), which contains 0.5% NaCl when prepared according to the manufacturer's directions. For studies involving

growth of the organism at elevated NaCl levels, quantities of NaCl were added to known volumes of TSB, and the percentage of NaCl was expressed on the basis of grams of NaCl per grams of final NaCl-TSB mixture, assuming the density of TSB to be 1.0. Each of the remaining solutes studied (KCl, glucose, sucrose, glycerol, and propylene glycol) were added individually to TSB containing 2.93% NaCl (TSBS) in progressively increasing amounts to achieve a_w values above and below which *V. parahaemolyticus* would grow. Percentages of these solutes, which are referred to throughout this report, were calculated as grams of solute per grams of final mixture (solute plus TSBS), again assuming the density of TSBS to be near 1.0. All growth media were adjusted to pH 7.8 by adding 2 N NaOH, dispensed into either 13 by 100 mm or 16 by 150 mm screw-cap tubes and sterilized at 121 C for 15 min. Random measurement of pH after sterilization revealed changes of no greater than ±0.2.

Growth studies. Since *V. parahaemolyticus* is facultatively halophilic, an initial experiment was designed to determine the optimal NaCl concentration at which the organism would grow. Strain M5250J-2 was cultured in TSBS at 29 C on a gyratory shaker (150 rpm) for 16 h. The culture was diluted 100-fold in distilled water containing 0.1% peptone (Difco) and 3.0% NaCl; 1 ml of the diluted culture was then inoculated into 150-ml portions of fresh TSB containing concentrations of NaCl ranging to 7.87%. The 500-ml Erlenmeyer flasks containing the cultures were returned to the shaker at 29 C. Samples were withdrawn at selected times and appropriate dilutions were made in the peptone-NaCl diluent prior to surface-plating on TSBS containing 1.5% agar (TSBSA) and on thiosulfate citrate bile salts sucrose agar (TCBS). Counts were made after 12 h (TSBSA) and 24 h (TCBS) of incubation at 36 C, and generation times were calculated for the organism cultured in each of the TSB media containing added NaCl.

The above experiment showed strain M5250J-2 to have the fastest generation time in TSB containing 2.93% NaCl (TSBS). Addition of various quantities of NaCl, KCl, glucose, sucrose, glycerol, or propylene glycol to TSBS was made to achieve only slight reductions in a_w levels. The effects of these a_w reductions on lag times of strain M5250J-2 were then examined by measuring absorbance of the growing cultures at 620 nm after inoculation with a loop of 16-h TSBS culture. Growth temperature for the inoculum and the test cultures was 36 C. Amounts of solutes added, their weight percents, and resulting a_w are summarized in Table 1.

Minimal a_w for growth of each of four strains of *V. parahaemolyticus* at 15, 21, 29, and 36 C were determined. Each strain was cultured in TSBS for 16 h at 29 C and standard loop inocula were transferred to 10-ml portions of TSBS containing individually added quantities of test solutes. Each test was performed in quintuplicate. In all cases, sufficient solute was added to achieve several a_w levels both above and below that required for growth. Caps were tightened on the tubes (16 by 150 mm), and the inoculated media were incubated for 20 days in walk-in incubators adjusted to 15, 21, 29, and 36 ± 0.2 C. Obvious turbidity during the 20-day period was recorded as positive growth, and tubes were discarded. After 20 days all remaining tubes were examined for number of viable cells by plating on TCBS and for number of total cells by using a Petroff-Hauser counting chamber. Tubes were recorded as negative if viable cell population and direct microscope counts per milliliter were both less than the original number of cells per milliliter of culture at the time of inoculation. Direct counts were necessary to determine whether cell division was followed by death of significant numbers of cells during the 20-day incubation period.

Determination of a_w. Equilibrium relative humidity measurements were made at 29 C with Hygrosensor elements (no. 4-4822; Hygrodynamics, Inc., Silver Spring, Md.) mounted in lids of 8-oz jars. Hygrometer sensors were attached to a Hygrometer Indicator (model no. 4-4900; Hygrodynamics, Inc.) and an equilibration time of not less than 8 h was allowed before measurements were recorded from 25-ml portions of each test solution. Sensory calibrations were made against saturated KNO_3. Mean values were determined from triplicate readings for several concentrations of each solute and sorption isotherms were plotted (Fig. 1). All a_w levels reported in this paper were taken from isotherm curves. Accuracy of the measuring system is considered to be no better than ±0.005 a_w. However, for purposes of comparison, as will become evident upon examination of results, data are presented at the ±0.001 a_w level.

RESULTS AND DISCUSSION

Inhibitory effects of a_w levels higher and lower than optimum on the growth and metabolism of several bacterial species have been reported (3, 5, 6, 16, 17). Extreme sensitivity to relatively minor variations in osmotic and ionic conditions in growth media have been most dramatic with *V. costicolus* (8) and *V. metchnikovi* (13). Reduction in a_w from 0.999 to 0.995 resulted in a fivefold increase in the rate of growth of *V. metchnikovi*. Further reduction in a_w generally resulted in decreased rates of growth, depending upon the solute added to control a_w and the nutrient composition of the growth medium. Several reports indicate conflicting ranges and optima for percentage of NaCl tolerance for *V. parahaemolyticus* (11, 21, 22, reviewed by 14; C. R. Lazarus and J. A. Koburger, Abst. Southeastern Branch Amer. Soc. Microbiol., 51st and 52nd Ann. Mtg., p. 19, 1973). It is difficult to compare these data because methods for preparing "percent NaCl" levels in media were not detailed and a_w levels were not reported. Initial experiments were therefore designed to determine the optimal NaCl concentration (and corresponding a_w) for growth of *V. parahaemolyticus* by using TSB as a basal medium. Results are shown in Fig. 2. As a_w was decreased from 0.998 to 0.992 (0.5 and 2.93% NaCl, respectively), the generation time of *V.*

TABLE 1. *Effect of added solutes on a_w of TSB and TSBS*

Solute	g of Solute added per 100 ml		Calculated solute concn (% wt)	a_w[a]
	TSB	TSBS		
NaCl	0		0.50	0.998
	2.5		2.93	0.992
	4.5		4.78	0.984
	5.5		5.69	0.979
	7.0		7.01	0.971
KCl		1.0	0.99	0.990
		2.5	2.44	0.986
		4.0	3.85	0.980
		5.5	5.21	0.974
Glucose		2.0	1.96	0.990
		4.0	3.85	0.989
		6.0	5.66	0.987
Sucrose		10.0	9.09	0.988
		20.0	16.67	0.982
		30.0	23.08	0.975
		40.0	28.57	0.968
Glycerol		3.0	2.91	0.988
		9.0	8.29	0.977
		14.0	12.28	0.968
		18.0	15.25	0.961
Propylene glycol		4.0	3.85	0.990
		6.0	5.66	0.989
		8.0	7.41	0.987

[a] a_w levels are listed for growth curves shown in Fig. 3; levels were calculated from sorption isotherms shown in Fig. 1.

parahaemolyticus M5250J-2 decreased from 24.4 to 16.4 min. Increased NaCl levels above 2.93% prolonged generation times. Whether a_w manipulation by the addition of other electrolytes or nonelectrolytes alone or in combination to TSB would have resulted in different a_w optima and faster growth rates was not determined. The 16.4-min generation time was considered to result from nearly optimal culture conditions, and therefore TSBS (TSB containing 2.93% NaCl) was used as a basal medium in subsequent studies involving the effects of added solutes on lag phase extension and minimal a_w tolerance of the four test strains.

Realizing that only slight differences in a_w achieved from the addition of NaCl to the growth medium resulted in substantial differences in generation times of strain M5250J-2, it was decided to measure the effects of low amounts of added KCl, glucose, sucrose, glycerol, and propylene glycol, in addition to NaCl, on lag times of the organism. Data are plotted in Fig. 3. Grams of solute added per 100 ml of TSB or TSBS, calculated solute concentrations, and corresponding a_w levels are summarized in Table 1. Some trends can be observed in these data. NaCl studies show that 0.992 a_w results in the shortest lag time, fastest growth rate, and highest total biomass production. Departure from 0.992 a_w resulted in longer lag times with slower growth rates and depressed cell production. These data tend to confirm those derived from generation time studies. Addition of solutes to TSBS (0.992 a_w) reduced a_w in all cases, but the magnitude of change in growth parameters was varied, depending upon the solute added. Glycerol had the least inhibitory effect on *V. parahaemolyticus* of all solutes tested. Addition of glycerol to achieve a 0.961 a_w

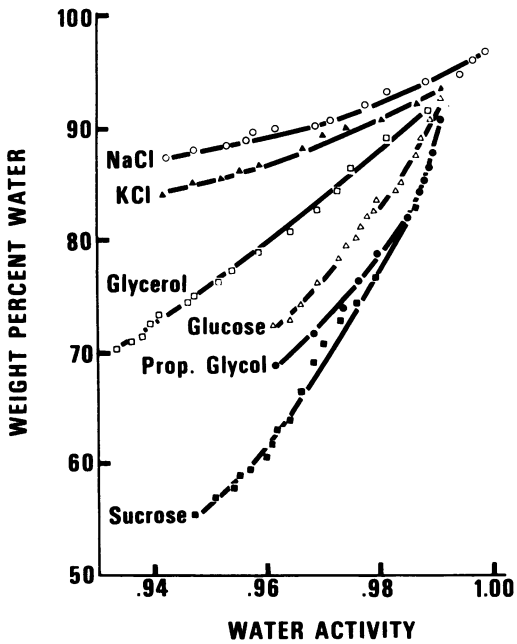

FIG. 1. *Sorption isotherms for TSB and TSBS basal media containing added solutes.*

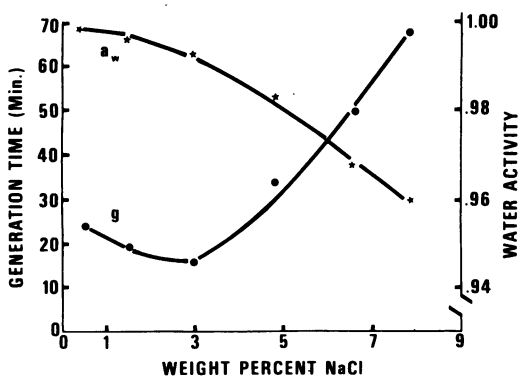

FIG. 2. *Effect of NaCl concentration and corresponding a_w on generation times of V. parahaemolyticus M5250J-2.*

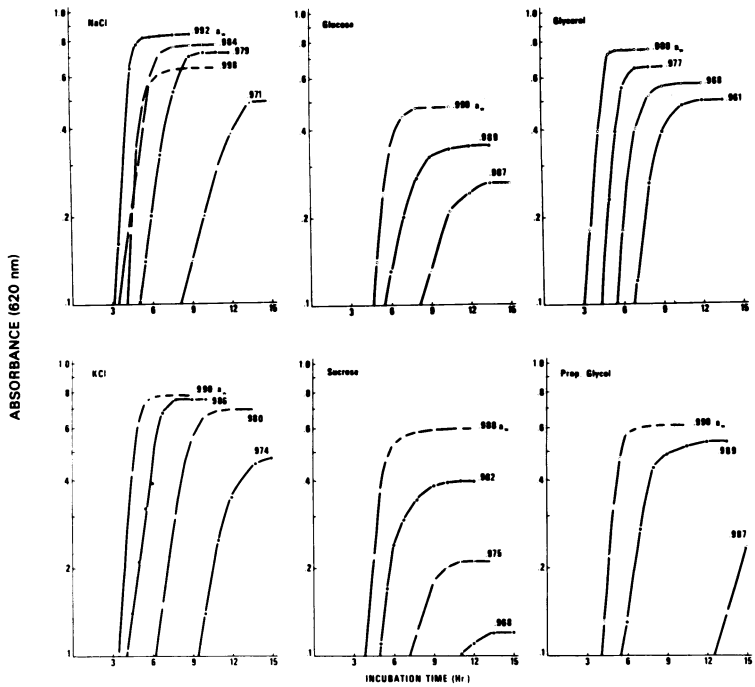

FIG. 3. *Effect of a_w on the growth of V. parahaemolyticus M5250J-2. See Table 1 for summary of media preparation.*

extended the lag phase only by 4 h compared to greater lag phase prolongation at even higher a_w levels for the other test solutes. Inhibitory effects of NaCl and KCl were similar at comparable reduction in a_w levels, whereas propylene glycol, glucose, and sucrose, in that general order, were most effective in delaying logarithmic growth and depressing cell production by strain M5250J-2. The glycerol data are in agreement with those reported for *Staphylococcus aureus* survival (15) and enterotoxin production (19) and growth of *Salmonella oranienburg* (4), *Bacillus cereus* (9), and *Clostridium perfringens* (10), wherein glycerol was found to be less inhibitory than other test solutes. Marshall et al. (12), on the other hand, reported that at a_w levels between 0.96 and 0.90 the inhibition of *S. aureus* growth rate was about 10% greater in glycerol than in NaCl. Glucose and propylene glycol proved to be most bacteriostatic to *V. parahaemolyticus* when compared to the other solutes at similar a_w levels. Total inhibition of growth of *V. metchnikovi* in nutrient broth containing glucose in amounts to achieve a_w less than 0.997 has been noted (13). These authors reported no growth of the organism in brain heart infusion broth containing glucose. Glucose appears, therefore, to exhibit a toxic effect on *V. metchnikovi* and *V. parahaemolyticus*. By-products arising from chemical reactions between glucose and medium constituents during sterilization may account for these inhibitors. The reasons for *V. parahaemolyticus* sensitivity to propylene glycol cannot be explained.

Table 2 shows the minimum a_w for growth of *V. parahaemolyticus* in TSB and TSBS adjusted to reduced a_w levels by the addition of various solutes. Calculated concentrations of added solutes are also listed. The four test strains responded similarly in their minimal a_w levels at various temperatures. In most cases the a_w levels listed in Table 2 are representative of at least three of the four strains. Therefore, only one minimal a_w is listed for each solute-temperature combination. In general, solutes which were more inhibitory with respect to prolonging lag phase of growth were also more effective in completely inhibiting growth. Glycerol was least effective in controlling growth, followed by NaCl and KCl which were approximately equal, and then sucrose, glucose, and propylene glycol. The 29 C incubation temperature proved to be most satisfactory for the organism's tolerance to low a_w, whereas 15 C adversely effected the response to a_w stress. *V. metchnikovi* was reported to have lower tolerance to glycerol than to NaCl when a_w adjustment was made in quarter-strength brain heart

TABLE 2. *Minimal a_w levels for growth of V. parahaemolyticus*

Solute	Incubation temp (C)	Minimal a_w[a] for growth	g of Solute added per 100 ml		Calculated solute concn (% wt)
			TSB	TSBS	
NaCl	15	0.962	8.6		8.38
	21	0.951	10.2		9.71
	29	0.948	10.5		9.95
	36	0.954	9.9		9.46
KCl	15	0.964		8.0	7.41
	21	0.956		9.5	8.67
	29	0.945		11.3	10.15
	36	0.958		9.0	8.26
Glucose	15	0.984		10.0	9.09
	21	0.983		11.0	9.91
	29	0.984		10.0	9.09
	36	0.984		10.0	9.09
Sucrose	15	0.967		42.0	29.58
	21	0.960		52.5	34.43
	29	0.957		57.0	36.31
	36	0.957		57.0	36.31
Glycerol	15	0.950		24.5	19.68
	21	0.946		27.0	21.26
	29	0.937		32.0	24.24
	36	0.942		29.0	22.48
Propylene glycol	15	0.987		12.0	10.71
	21	0.987		12.5	11.11
	29	0.986		13.5	11.89
	36	0.988		10.5	9.50

[a] Calculated from sorption isotherms, shown in Fig. 1.

broth (12). Although the source of broth was not stated by the authors, if a Difco or BBL product was used, the initial concentration of NaCl was 0.5%. In view of the relatively high ionic strength required in growth media by *Vibrio* spp., the reversal in apparent tolerance of *V. metchnikovi* and *V. parahaemolyticus* to NaCl and glycerol might partially be explained on the basis of electrolyte concentration of the basal media used in the two experiments. The TSBS basal medium used to establish solute tolerances in the present study may have provided sufficient electrolyte to satisfy *V. parahaemolyticus* requirements. Therefore, addition of a nonelectrolyte such as glycerol to TSBS may have resulted in minimal a_w levels which measured tolerance to the nonelectrolyte instead of tolerance to a combination of stresses induced from low electrolyte and high nonelectrolyte concentrations concurrently.

The relationship between limiting a_w levels for growth of microorganisms and the solute added to achieve those levels is unclear. Several authors (7, 17) have stated that biological response to a_w by some organisms is independent of the types of solutes used to reduce the a_w. Other reports have shown that nutrient availability (3), pH (1), oxygen level (6, 16), and moisture content (15), in addition to the test solute, effect a microorganism's ability to grow at limiting a_w. It appears that the limiting a_w for growth of the four *V. parahaemolyticus* strains examined in this study depends upon the solutes used to attain these lower limits. Notwithstanding the possibility of nutrient dilution effects inherent in the methods employed to prepare the test media, the differences in physico-chemical properties of the solutes apparently have a significant influence on the ability of *V. parahaemolyticus* to tolerate suboptimal a_w levels. Further experiments are required to determine the response of *V. parahaemolyticus* to low a_w levels achieved by the addition of other halogen salts to growth media. Studies involving the combined effects of various electrolytes and nonelectrolytes on tolerance of the organism at reduced a_w would also provide valuable information on growth characteristics of this facultative halophile.

ACKNOWLEDGMENT

The technical assistance of B. Vaughn is gratefully acknowledged.

LITERATURE CITED

1. Baird-Parker, A. C., and B. Freame. 1967. Combined effect of water activity, pH and temperature on the growth of *Clostridium botulinum* from spore and vegetative cell inocula. J. Appl. Bacteriol. **30**:420-429.
2. Center for Disease Control. 1973. Morbidity and Mortality Weekly Rept. **22**:231-232.
3. Christian, J. H. B. 1955. The influence of nutrition on the water relations of *Salmonella oranienburg*. Aust. J. Biol. Sci. **8**:75-82.
4. Christian, J. H. B. 1955. The water relations of growth and respiration of *Salmonella oranienburg* at 30°C. Aust. J. Biol. Sci. **8**:490-497.
5. Christian, J. H. B., and J. A. Waltho. 1962. The water relations of staphylococci and micrococci. J. Appl. Bacteriol. **25**:369-377.
6. Christian, J. H. B., and W. J. Scott. 1953. Water relations of salmonellae at 30°C. Aust. J. Biol. Sci. **6**:565-573.
7. Emodi, A. S., and R. V. Lechowich. 1969. Low temperature growth of type E *Clostridium botulinum* spores. 2. Effects of solutes and incubation temperature. J. Food Sci. **34**:82-87.
8. Flannery, W. L., R. N. Doetsch, and P. A. Hansen. 1952. Salt desideration of *Vibrio costicolus*, an obligate halophilic bacterium. I. Ionic replacement of sodium chloride requirement. J. Bacteriol. **64**:713-717.
9. Jakobsen, M., O. Filtenborg, and F. Bramsaes. 1972. Germination and outgrowth of the bacterial spore in the presence of different solutes. Lebensm.-Wiss. u. Technol. **5**:159-162.
10. Kang, C. K., M. Woodburn, A. Pagenkopf, and R. Cheney. 1969. Growth, sporulation, and germination of

Clostridium perfringens in media of controlled water activity. Appl. Microbiol. **18**:798-805.
11. Lee, J. S. 1973. What seafood processors should know about *Vibrio parahaemolyticus*. J. Milk Food Technol. **36**:405-408.
12. Marshall, B. J., D. F. Ohye, and J. H. B. Christian. 1971. Tolerance of bacteria to high concentrations of NaCl and glycerol in the growth medium. Appl. Microbiol. **21**:363-364.
13. Marshall, B. J., and W. J. Scott. 1958. The water relations of *Vibrio metchnikovi* at 30°C. Aust. J. Biol. Sci. **11**:171-176.
14. Nickelson, R., and C. Vanderzant. 1971. *Vibrio parahaemolyticus*—a review. J. Milk Food Technol. **34**:447-452.
15. Plitman, M., Y. Park, R. Gomez, and A. J. Sinskey. 1973. Viability of *Staphylococcus aureus* in intermediate moisture meats. J. Food Sci. **38**:1004-1008.
16. Scott, W. J. 1953. Water relations of *Staphylococcus aureus* at 30°C. Aust. J. Biol. Sci. **6**:549-564.
17. Scott, W. J. 1957. Water relations of food spoilage microorganisms. Advan. Food Res. **7**:83-127.
18. Strong, D. H., E. F. Foster, and C. L. Duncan. 1970. Influence of water activity on the growth of *Clostridium perfringens*. Appl. Microbiol. **19**:980-987.
19. Troller, J. A. 1971. Effect of water activity on enterotoxin B production and growth of *Staphylococcus aureus*. Appl. Microbiol. **21**:435-439.
20. Troller, J. A. 1973. The water relations of food-borne bacterial pathogens. A review. J. Milk Food Technol. **36**:276-288.
21. Twedt, R. M., P. L. Spaulding, and H. E. Hall. 1969. Morphological, cultural, biochemical, and serological comparison of Japanese strains of *Vibrio parahaemolyticus* with related cultures isolated in the United States. J. Bacteriol. **98**:511-518.
22. Zen-Yoji, H., H. Hitokoto, S. Morozumi, and R. A. LeClair. 1971. Purification and characterization of a hemolysin produced by *Vibrio parahaemolyticus*. J. Infect. Dis. **123**:665-667.