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Resistance of Cold- and Starvation-Stressed *Vibrio vulnificus* to Heat and Freeze-Thaw Exposure

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ABSTRACT

The effects of cold storage and starvation on the subsequent heat resistance and freeze-thaw resistance of *Vibrio vulnificus* were studied. Three strains of *V. vulnificus* were evaluated. Cold stress had no effect on freeze-thaw resistance ($P > 0.05$). Starvation enhanced freeze-thaw resistance for one strain compared to controls ($P < 0.05$). *V. vulnificus* was not heat resistant; control populations were inactivated within 12 min at 47°C. Starvation increased heat tolerance for one strain, but differences were small from a processing perspective ($P < 0.05$). Cold stress had no effect on heat resistance ($P > 0.05$). Cold adaptation (holding 4 h at 15°C) enhanced cold temperature (5°C) tolerance. This information will be helpful in the development of methods to minimize *V. vulnificus* risk.

Vibrio vulnificus is a human pathogen and is a gram-negative, rod-shaped halophilic bacterium. *V. vulnificus* was first reported as a serious human infectious bacterium in 1976 by Hollis et al. *(8).* Since first being reported, *V. vulnificus* has caused foodborne illness and wound infections throughout the world, including the United States *(16).* This estuarine bacterium is highly invasive, causing fulminate primary septicemia in persons at risk for infection, with a mortality rate of around 60% *(11, 26, 29, 30).* Infection resulting in primary septicemia is associated with the consumption of raw oysters and other shellfish *(9, 12).* Ninety-five percent of seafood-related deaths are due to *V. vulnificus (18).*

Numbers of *V. vulnificus* peak in seawater between May and October, and it is difficult to isolate the bacterium from seawater when water temperatures are below 10°C *(17).* High populations (10^3 to 10^4 most-probable-number populations per g) of *V. vulnificus* are found in Gulf Coast oysters during the summer (May to October), and numbers are reduced to 10 most-probable-number populations per g from December to March *(17).* The seasonal distribution of isolation of *V. vulnificus* is thought by some researchers to reflect entrance by this bacterium into a viable but nonculturable (VBNC) state. Cells in a VBNC state remain viable but no longer multiply or develop colonies on nonselective media. The VBNC condition of *V. vulnificus* may serve as a survival strategy against suboptimal environmental conditions. Although various gram-negative bacteria, including *E. coli, Salmonella* Enteritidis, *Shigella sonnei (4, 32, 33), Vibrio cholerae (33),* and *Legionella pneumophila (28),* were reported to enter a VBNC state, the existence of the VBNC state is controversial *(13),* and the enumeration of culturable cells continues to be the optimal method for assessing processing regimes and food safety risk.

Stress conditions may be encountered by bacteria in minimally processed foods *(1).* Poststress growth may occur during subsequent processing or handling steps. Food processing methods apply a variety of stresses analogous to environmental stresses that interfere with bacterial homeostasis, inhibit growth, or inactivate foodborne pathogens *(1).* A stress response may enable survival under more extreme conditions and enhance virulence and resistance to subsequent processing conditions *(2, 6, 10, 15).* Little research has addressed the potential for a stress response in *V. vulnificus.* A more complete understanding of the effects of sublethal stress on subsequent poststress resistance of *V. vulnificus* is important for the control of this pathogen. This study was undertaken to determine the effects of starvation and cold stress on poststress heat resistance, freeze-thaw resistance, and cold temperature adaptation of *V. vulnificus.*

MATERIALS AND METHODS

Cultures. *V. vulnificus* (ATCC 27562) was purchased from the American Type Culture Collection (Manassas, Va.). Two other *V. vulnificus* strains, 304C and MO6-24, were obtained from Dr. Angelo DePaola (U.S. Food and Drug Administration, Dauphin Island, Ala.). *V. vulnificus* strain 304C was isolated from oysters (Alabama). *V. vulnificus* strain MO6-24 was isolated from a primary septicemia case associated with raw oyster consumption. The three strains of *V. vulnificus* were confirmed by fatty acid membrane composition analysis *(24)* using gas chromatography (HP 6890, Hewlett Packard, Tex.).

Stock strains were maintained at room temperature on tryptic soy agar with 2% sodium chloride ($TSAN_2$) slants with sterilized mineral oil overlays (Sigma Chemical Co., St. Louis, Mo.). *V. vulnificus* stock strains were transferred monthly. Three consecutive transfers of each strain in tryptic soy broth with 2% sodium chloride ($TSBN_2$) at 37°C for 12 h were conducted before experimental use *(31).* A growth curve at 37°C in $TSBN_2$ was generated

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for each strain. Stationary phase was achieved within 12 h at 37°C in $TSBN_2$. On the basis of growth curve characteristic studies, the stationary phase of *V. vulnificus* in $TSBN_2$ was determined as 10^8 to 10^9 CFU/ml, and the optical density at 600 nm was found to be 0.35 to 0.38 (DU 530; Beckman, Fullerton, Calif.). Stationary-phase cells were used in subsequent studies.

Preparation of cells: control cells. Control cells were grown in $TSBN_2$ for 12 h at 37°C.

Preparation of cells: starved cells. One milliliter of starvation-stressed cells was prepared by centrifugation (10,000/rpm, 2 min) of a 12-h culture (grown in $TSBN_2$ to 10^8 CFU/ml), followed by a resuspension of the pelleted cells in 1 ml of 2% sodium chloride for 48 h at 37°C. Preliminary studies confirmed that viable cell counts for each strain decreased no more than 1 log after starvation stress.

Preparation of cells: cold-stressed cells. Cold-stressed cells were prepared by centrifugation (10,000/rpm, 2 min) of a 12-h culture (grown in $TSBN_2$ to 10^8 CFU/ml), followed by resuspension of the pelleted cells in 1 ml fresh $TSBN_2$ (21°C) and subsequent storage for 5 days at 5°C. Preliminary studies confirmed that viable cell counts for each strain decreased no more than 1 log after cold stress.

Growth characteristics of control and stressed cells. Control cells (10^7 to 10^8 CFU/ml) and stressed cells (10^6 to 10^7 CFU/ml) were diluted in $TSBN_2$ to 10^4 CFU/ml, and 1 ml of diluted cell suspension was transferred to 99 ml $TSBN_2$ and incubated at different temperatures (15, 21, and 37°C). Samples were taken at appropriate times for enumeration to assess growth characteristics (lag time and time to reach stationary phase). Samples were taken at 0, 2, 4, 8, 12, 16, and 24 h at 21 and 37°C. Samples were taken at 0, 2, 4, 12, 16, 24, 48, and 72 h at 15°C. Samples were pour plated in duplicate on $TSAN_2$ and incubated for 24 h at 37°C.

Freeze-thaw resistance. One milliliter of control or stressed cells was transferred to 1.5-ml microcentrifuge tubes, centrifuged, and resuspended in 1 ml of fresh $TSBN_2$. Cultures were subsequently stored at −20°C. One freeze-thaw cycle consisted of freezing at −20°C for 24 h, followed by thawing at 23°C for 30 min in air *(15).* Freeze-thaw cycles were repeated five times. Duplicate thawed samples (1 ml) were taken at each time point, diluted with sterile $TSBN_2$, and plated in duplicate on $TSAN_2$. The plates were incubated at 37°C for 24 h, and the total colonies were counted.

Heat tolerance. One milliliter of cells was transferred into 99 ml of preheated (47°C) sterile $TSBN_2$ in a water bath as described by Leenanon and Drake *(15).* Samples were withdrawn after heat treatment for 0, 3, 6, 9, 12, 15, and 18 min. Withdrawn samples were diluted with sterile $TSBN_2$ and plated in duplicate with $TSAN_2$. The plates were incubated at 37°C for 24 h.

Cold temperature adaptation. The ability of *V. vulnificus* to adapt to cold storage temperatures was also investigated. The three strains were grown at 37°C for 12 h to 10^8 CFU/ml (optical density at 600 nm of 0.35 to 0.38). One milliliter of cells was transferred into 99 ml sterile $TSBN_2$, prechilled at 5°C, and placed at 5°C. Samples were withdrawn after cold treatment (5°C) for 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 days. For cold temperature adaptation, cells were stored at 15°C for 4 h as described by Bryan et al. *(3)* before cold storage at 5°C. Withdrawn samples were diluted with sterile $TSBN_2$ and plated in duplicate with $TSAN_2$. The plates were incubated at 37°C for 24 h.

TABLE 1. D-*values in days for* V. vulnificus *(ATCC 27562, 304C, and MO6-24) after six freeze-thaw (−20°C/23°C) cycles*

Strains	Stress conditions		
	Control	Cold stress	Starvation
ATCC 27562	0.8 c[a]	0.9 c	1.6 A
304C	0.8 c	1.0 c	1.1 B
MO6-24	0.9 c	0.9 c	1.1 B

[a] Values followed by different letters are significantly different ($P < 0.05$). *D*-values are means of three replications. A significant interaction between strain and stress was observed ($P < 0.05$); thus, individual means are compared.

Statistical analysis. Three replications of each treatment were performed for each strain. *D*-values were calculated as the time in days or minutes required for a 1-log reduction of the population in each treatment. *D*-values were calculated by regression analysis (PROC REG). Analysis of variance of *D*-values was then conducted (PROC MIXED), and the least-square method was used to determine significant differences ($P < 0.05$) (SAS Statistical Analysis Software, version 8.0, SAS Institute, Cary, N.C.). When significant interactions for strain type and stress occurred, the strain-stress combinations were addressed and compared.

RESULTS AND DISCUSSION

Growth characteristics. Growth characteristics were affected by incubation temperature. However, there were no differences in growth characteristics between control cells and poststressed cells. All three *V. vulnificus* strains reached 10^8 CFU/ml after 12, 24, and 72 h at 37, 21, and 15°C, respectively, regardless of prior stress. The optimum growth of *V. vulnificus* was at 37°C. At 21 and 15°C, the strains continued to grow, although at a decreased rate. From preliminary studies, *V. vulnificus* strains did not grow above 44°C or below 13°C (data not shown). Kelly *(14)* reported that *V. vulnificus* had a maximum growth temperature of 37°C and that the organism also grew well at 30 and 35°C. However, no growth occurred at 13°C after a 12-h incubation *(14).*

Freeze-thaw resistance. In general, viable *V. vulnificus* decreased with the number of freeze-thaw cycles. Control strains were not significantly different in freeze-thaw resistance ($P > 0.05$). Cold storage (5°C for 5 days) did not have cross-protective effects on freeze-thaw resistance ($P > 0.05$) (Table 1 and Fig. 1A through 1C). The time required for *V. vulnificus* to die appreciably during cold storage appeared to exceed the cold storage of 14 days for shucked meats and 21 days for shell stock oysters *(18).* However, freezing temperatures contributed to a significant reduction in the numbers of *V. vulnificus (5).* Cook and Ruple *(5)* demonstrated that the viability of *V. vulnificus* was significantly decreased when oysters were stored on ice for 3 days. Our results showed that each strain of *V. vulnificus* declined with each freeze-thaw cycle. This observation was consistent with observations that the isolation of this bacterium decreases from seawater during winter months and with other previous research on cold storage of *V. vulnificus (5, 14).* Research with other bacteria has

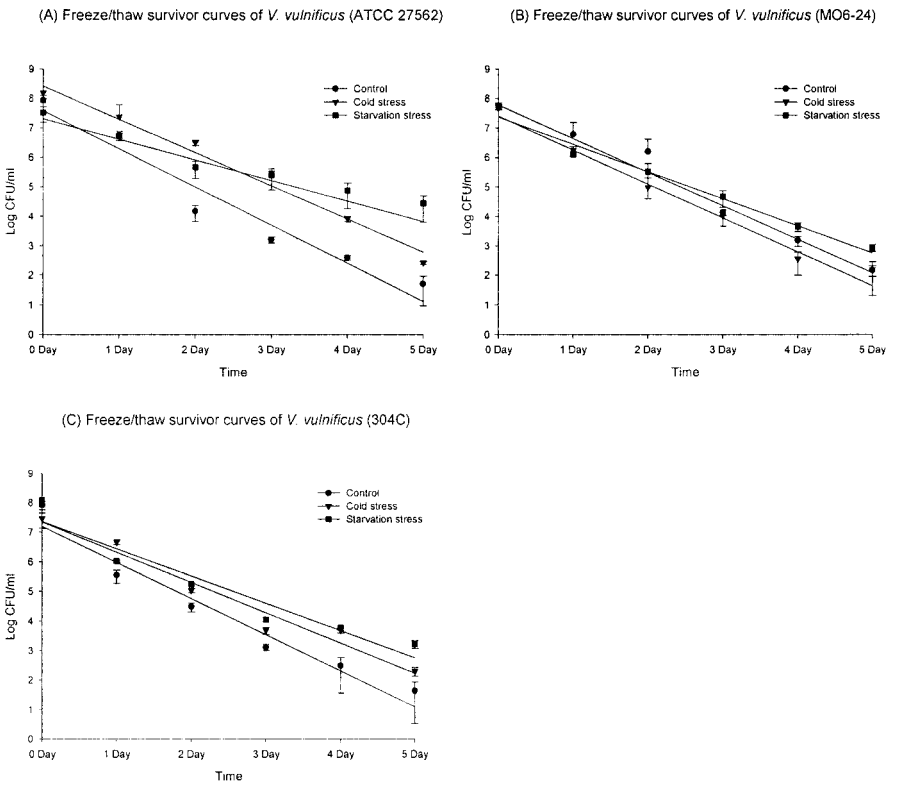

FIGURE 1. *Freeze-thaw survivor curves for* V. vulnificus *(A) ATCC 27562, (B) MO6-24, and (C) 304C. Bars represent the standard error from three replications.*

shown that cold stress can increase freeze-thaw resistance *(15, 22).* Bryan et al. *(3)* reported that cold adaptation at 15°C before storage at 6°C increased the survival of *V. vulnificus* from −78°C frozen storage. An examination of protein production indicated the possibility of cold adaptation (15°C)–induced proteins. These authors suggested that survival and tolerance at cold and freezing temperatures could be due to the expression of cold-adaptive proteins rather than to cold-shock proteins. One strain was used in the study, and freeze-thaw (−20/23°C) resistance was not addressed. Doubtless, strain differences exist among *V. vulnificus,* and future work should address the topic of cold adaptation in the range of 7 to 15°C and its effects on cross-adaptation to food processing and storage conditions.

Starvation (37°C for 48 h) increased freeze-thaw resistance ($P < 0.05$) for all three strains, although results were significant only from a food processing perspective with one strain (ATCC 27562) (Table 1 and Fig. 1A through 1C). Leenanon and Drake *(15)* also noted enhanced freeze-thaw resistance of *E. coli* after starvation. Starvation was reported to enhance the survival of *V. vulnificus* at cold temperatures *(21).*

TABLE 2. D-*values in minutes for* V. vulnificus *(ATCC 27562, 304C, and MO6-24) in TSBN$_2$ at 47°C after starvation stress*

Strains	Stress condition	
	Control	Starvation
ATCC 27562	1.8 B[a]	1.9 B
304C	1.7 B	1.8 B
MO6-24	1.9 B	2.4 A

[a] Values followed by different letters are significantly different ($P < 0.05$). *D*-values are means of three replications. A significant interaction between strain and stress was observed ($P < 0.05$); thus, individual means are compared.

Heat tolerance. The three strains were not heat resistant, and $D_{47°C}$-values were less than 2 min (Table 2). Initial populations (5 to 6 $\log_{10}$) were inactivated within 12 min at 47°C (Fig. 2A through 2C). After 10 min at 50°C, *V. vulnificus* was not detected in oysters *(5).* Shultz et al. *(27)* reported that thermal death characteristics of *V. vulnificus* were similar to other pathogenic vibrios. *V. cholerae* had D_{49}- and D_{54}-values of 102 and 62.4 s, respectively. Also, *Vibrio parahaemolyticus* had D_{47}- and D_{50}-values of 113.8 and 39.8 s, respectively. Our results were consistent with these findings.

Thermal survivor curves were biphasic. Peleg and Cole *(23)* reported biphasic death curves with *V. vulnificus.* Reported *D*-values were calculated on the basis of initial log linear death rate. Starvation increased heat tolerance for strain MO6-24 ($P < 0.05$) (Table 2 and Fig. 2A through 2C), but the difference was probably not significant from a food processing perspective. Heat tolerance was not affected by cold stress ($D_{47} = 2.0 \pm 0.1$ min) ($P > 0.05$). Bacteria grown at low temperatures may incorporate more unsaturated fatty acids into their cell membranes to maintain functional membrane fluidity and, as a result, decreased heat resistance may occur because of the reduced melting point of unsaturated acids within cell membranes *(25).* Leenanon and Drake *(15)* reported that heat resistance was decreased in cold-stressed *E. coli* cells. *V. vulnificus* did not exhibit an increase in heat sensitivity after cold stress. This characteristic may be because of their extreme heat sensitivity compared to other gram-negative foodborne pathogens.

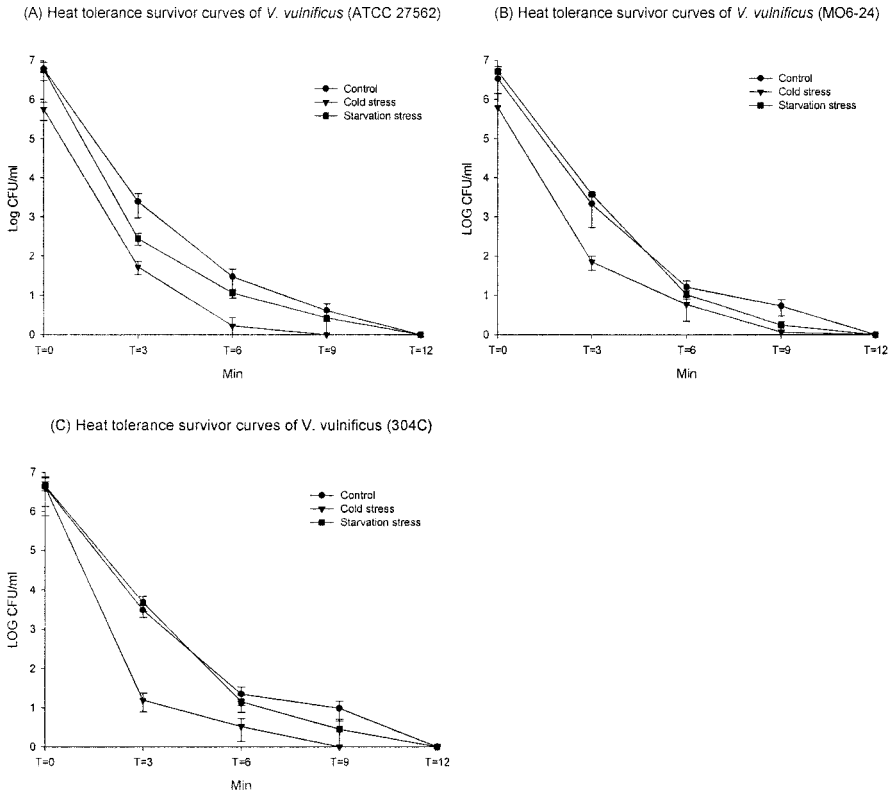

FIGURE 2. *Thermal inactivation survivor curves for* V. vulnificus *(A) ATCC 27562, (B) MO6-24, and (C) 304C at 47°C. Bars represent the standard error from three replications.*

Cold temperature adaptation. When *V. vulnificus* cells were transferred from 37 to 5°C, culturable cell numbers decreased (Fig. 3A through 3C). Cold-adapted cells were more tolerant to cold temperatures than control cells. The effects of cold adaptation varied among the three strains. The protective effects of cold adaptation were more enhanced in ATCC 27562 than in the other two strains (Fig. 3A through 3C). Bryan et al. *(3)* also reported that cold adaptation with one *V. vulnificus* strain increased survival more than 2 $\log_{10}$ populations compared to direct cold storage survival and suggested that cold-adaptive protective proteins might enhance survival and tolerance at cold temperatures. The decrease in viable cells upon cold storage may represent the transition to a VBNC state, but the evaluation of VBNC cells was not an objective of this study. Although *V. vulnificus* cells in a VBNC have been shown to retain virulence in mice *(20),* the methodology and their exact public health significance remain debatable. The current study focused on evaluating *V. vulnificus* stress response and viability after stress using traditional cultural methods.

Cell death (or loss of culturability) at 5°C differed noticeably between cold stress experiments and cold temperature adaptation experiments. There was little cell death at 5°C across 5 days' storage in cold stress experiments (<1 $\log_{10}$ for each strain), whereas viable control cells decreased by 2 to 4 $\log_{10}$ depending on the strain during 5 days at 5°C for cold adaptation experiments. This discrepancy was puzzling at first but may be explained upon noting how cells were treated at cold storage. For cold stress experiments, cells were resuspended in room temperature $TSBN_2$ and subsequently chilled to 5°C and stored. Control cells in cold adaptation experiments were resuspended directly in prechilled (5°C) $TSBN_2$. It seems likely that cells resuspended in room temperature $TSBN_2$ had the opportunity to undergo cold adaptation during the time required for the broth to cool to 5°C and thus exhibited enhanced survival at 5°C. There were other differences between cold stress and cold adaptation experiments that also could have influenced observed cell survival. Cells treated to cold stress were washed before stress, and their numbers (initial colony-forming units) during cold storage were higher because of a smaller volume used. Temperature was reported to be one of the most important factors in *V. vulnificus* survival *(7),* but other factors including cell numbers and cell washing were also noted to influence the survival and culturability of *V. vulnificus (19).* These results further emphasize the need for a meticulous reporting of cell preparation and treatments in *V. vulnificus* studies, as small changes can result in large differences in observed results.

CONCLUSIONS

There were no differences in growth characteristics between control and stressed cells. Cold stress had no cross-protective effect on freeze-thaw resistance. Starvation stress had a cross-protective effect on freeze-thaw resistance for all three strains, but differences were significant only from a food processing perspective for one strain. Differences were observed for one strain with starvation and subsequent heat tolerance, but these were small from a food processing viewpoint. Cold or starvation stress of *V. vulnificus* did not appear to significantly affect heat or freeze-thaw tolerance from a food processing perspective. Cold adaptation did enhance cold storage survival. The effectiveness of cold temperatures to reduce *V. vulnificus* population may be

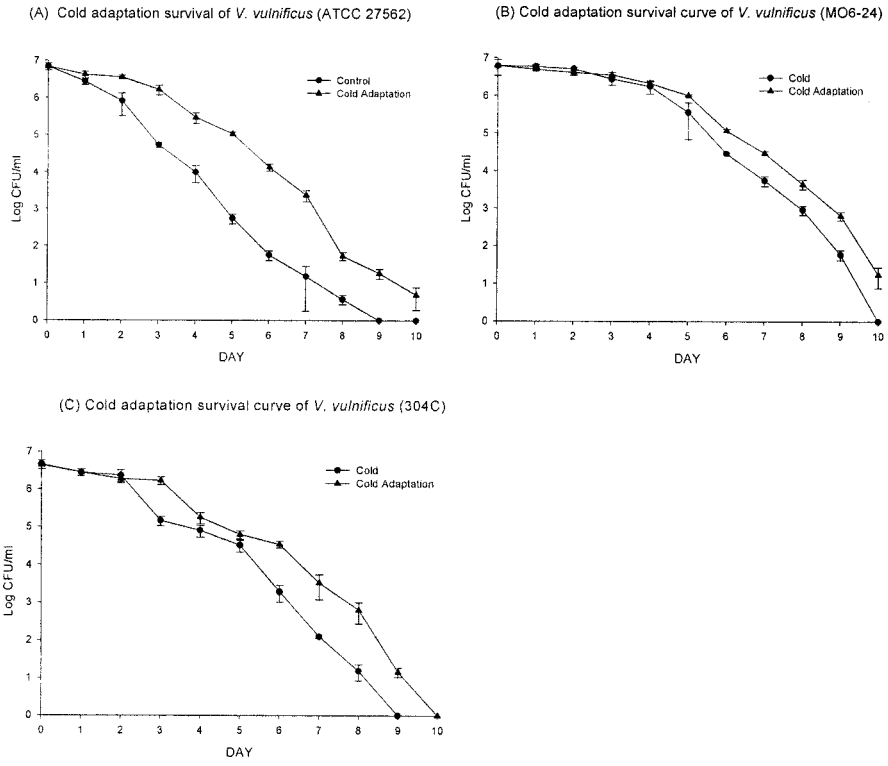

FIGURE 3. *Cold temperature inactivation comparison between control and cold-adapted cell of* V. vulnificus *(A) ATCC 27562, (B) MO6-24, and (C) 304 C at 5°C. Bars represent the standard error from three replications.*

compromised without prompt and thorough chilling of the food product.

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