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Lethal Cold Stress of *Vibrio vulnificus* in Oysters

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Studies were conducted on the survival of *Vibrio vulnificus*, an estuarine human pathogen, in oyster homogenates held at 4°C. Results indicated a rapid and dramatic decrease in viability not attributable to either cold shock or the oyster homogenate alone but to a combination of the two. Such a decline was not observed with *Vibrio parahaemolyticus*. Chilled *V. vulnificus* cells were unable to repair themselves in brain heart infusion broth at 37°C. *V. vulnificus* cells incubated on whole raw oysters at 0.5°C also exhibited a decline in viability, but of a lesser degree. The effects of various plating media were also investigated. The data reported here suggest that oysters kept on ice are not likely to be a major factor in the epidemiology of *V. vulnificus* infection. It is further suggested that the standard method of homogenizing oysters for examining bacteriological quality should not be followed because toxic compounds are released from the oysters during this process.

The lactose-fermenting halophile, *Vibrio vulnificus*, is a bacterium of exceptional virulence. This estuarine organism, which is differentiated taxonomically from *Vibrio parahaemolyticus* primarily by its ability to ferment lactose (8), is capable of causing death in laboratory animals in less than 3 h when injected intraperitoneally (19). Poole and Oliver (19) have demonstrated that *V. vulnificus*, unlike *V. parahaemolyticus* or *Vibrio cholerae*, also can cause death when inoculated subcutaneously. By either route, the cells appear to produce an extremely potent vascular permeability factor which leads to dramatic vascular fluid loss in laboratory animals (M. D. Poole, J. D. Oliver, and J. H. Bowdre, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, B15, p. 18). Light and electron microscopic studies of rabbit intestinal tissue after injection of *V. vulnificus* into ligated ileal loops revealed invasion of the submucosa followed by bacteremia and death of the host (J. D. Dellinger, M.S. thesis, University of North Carolina at Charlotte, Charlotte, 1980). These observations mimic the results observed in persons infected with the bacterium after ingestion of contaminated seafood (3). More specifically, the Centers for Disease Control in Atlanta, Ga., have reported a significant correlation between the ingestion of raw oysters and fatal infections (3). To date, however, the presence of *V. vulnificus* in oysters has not been established. Because large numbers of *V. parahaemolyticus* cells are known to frequently occur in fresh oysters (1) and in shellfish in general (22), the role of oysters in the epidemiology of *V. vulnificus* infections may be extremely important. Because raw oysters are generally eaten "on the half-shell" served on ice, it seemed appropriate to examine whether *V. vulnificus*, like many other bacteria (9, 18, 25), might undergo cell damage upon chilling and to determine what role oysters might play in the survival of this bacterium under these conditions.

MATERIALS AND METHODS

Bacteria. The following *V. vulnificus* strains were obtained from the Centers for Disease Control: C7184, A8694, H3308, A1402, and D4473. The *V. parahaemolyticus* strain used was ATCC 27519. All cultures were maintained on salt water-based agar slants (17).

Whole oysters and oyster broth. Fresh, raw oysters were purchased from a local market. For homogenates, oysters were added to a final concentration of 1% (wt/vol) in an estuarine salts (ES) solution (12.36 g of NaCl, 0.34 g of KCl, 0.68 g of $CaCl_2 \cdot 2H_2O$, 2.33 g of $MgCl_2 \cdot 6H_2O$, 3.15 g of $MgSO_4 \cdot 7H_2O$, 0.09 g of $NaHCO_3$, per liter of distilled water). The mixture was homogenized for 1 min in a Waring blender, dispensed in 100-ml portions to 250-ml screw-cap flasks, and sterilized at 121°C for 15 min. This procedure provided a medium of pH 7.85. For whole oyster studies, individual oysters were placed in sterile 50-ml screw-cap tubes and kept on ice.

Cold shock studies. Flasks of oyster broth prechilled to 4 ± 0.5°C or intact oysters on ice (temperature of oysters, 0.5°C) were inoculated with 1 ml of a culture of the *Vibrio* species to be studied grown to stationary phase in standing culture overnight at 23°C in ES broth (1 g of yeast extract–1 g of Proteose peptone [Difco Laboratories] per liter of ES; final pH, 7.3). Final cell concentrations were ca. 10^6 cells per ml of oyster broth or ca. 10^4 or 10^6 cells per oyster during whole oyster studies. At intervals, aliquots of oyster broth or oyster mantle fluid (in the case of whole oysters) were removed, diluted in ES, and plated to brain heart infusion agar (BHI; BBL Microbiology

Systems), BHI plus 2.5% NaCl (BHI+S), ES agar, or thiosulfate-citrate-bile salts-sucrose agar (TCBS; BBL Microbiology Systems). Plates were incubated overnight at 37°C.

Recovery (injury repair) studies. Recovery studies of cold-shocked *Vibrio* species were performed by removing, at various times, 1 ml of the inoculated oyster broth to 9 ml of BHI broth. The BHI broth recovery tube was maintained at 37°C for 60 min. At that time the broth was diluted in ES, plated to BHI, BHI+S, and TCBS, and incubated at 37°C overnight.

RESULTS

Survival of *V. vulnificus* in oyster broth. Incubation of *V. vulnificus* in oyster broth at 4°C produced a rapid and steady decline in viability, as indicated by growth on BHI (Fig. 1). These results were consistent for all five strains examined. Although several strains survived until the 24-h sampling (see slopes of the death curves), it seems likely that sterilization of all cultures occurred considerably before that time. On several occasions, survival times were shorter than those shown in Fig. 1. Although growth of *V. vulnificus* occurs only at temperatures above 12 to 13°C (unpublished observations), little decrease in viability was observed over the same time period when *V. vulnificus* was suspended in ES at 4°C. Unlike the *V. vulnificus* strains examined, *V. parahaemolyticus* exhibited little decline in survivability over the 24-h period when incubated at 4°C in oyster broth.

Effect of plating medium on survival assays. For investigating the possibility that a plating medium other than BHI might prove more suitable for these studies, aliquots of inoculated oyster broth were plated onto TCBS, ES agar, and BHI+S, as well as onto BHI. Figure 2 shows that TCBS, ES agar, and BHI all produced comparable results when used for either *V. vulnificus* or *V. parahaemolyticus*. The addition of salt to BHI (final concentration, 3%), however, greatly affected the survivability of both species. *V. vulnificus* was seen to be especially sensitive to elevated salt concentrations after cold shock, with strain C7184 unable to grow on the elevated salt medium even after only 24 min in oyster broth at 4°C. Although the data shown for *V. vulnificus* were the most dramatic observed, a similar pattern was seen in all other experiments on the effects of salts. *V. parahaemolyticus* also demonstrated a sensitivity to increased salt levels in the plating medium, with no cells detectable at the 24-h sampling,

Recovery (injury repair) studies. For examining whether the rapid decline observed in populations of the *V. vulnificus* cultures resulted from actual viability losses or was due to cold shock-injured cells that were subsequently unable to repair themselves and develop into colonies on the media used, a recovery study was carried out. For this purpose, a nutritionally rich, nonselective liquid medium (BHI broth) was employed, with a 60-min incubation period at 37°C to allow repair of any cold shock-induced cell damage. Although the BHI used as a plating medium in the previous experiments should have been totally nonselective and non-inhibitory, the possibility of some effect on plating due to surface tension or other phenomena could have been a factor in colony development. Results of this study are presented in Table 1. It is evident from these data that the injury sustained by the *V. vulnificus* cells held in oyster broth at 4°C was substantial and apparently precluded repair, at least under the conditions provided in this study. The same results were obtained when repair was attempted for 17 h in BHI broth at 25°C. Table 1 shows again the detrimental effect of additional NaCl in the plating medium, with BHI+S providing 1 log fewer cells after 6.5 h of incubation in oyster broth.

Effect of temperature on *V. vulnificus* survival in oyster broth. As seen in Fig. 3, no appreciable loss of viability of *V. vulnificus* occurred at 4°C in buffered salt solution alone. The oyster broth alone was also not lethal when the incubation temperature was maintained at 25°C. In fact, growth of *V. vulnificus* occurred under these conditions. Only when the cells were incubated in the chilled (4°C) homogenate was the rapid decline in viability observed, with a loss in excess of 5 logs during the 24-h period.

Survival of *V. vulnificus* on whole oysters. When *V. vulnificus* at cell concentrations of 3×10^6 to 4×10^6 cells per oyster (comparable to the concentrations used for the oyster homogenate studies) was inoculated onto whole oysters held at 0.5°C on ice, a gradual die-off was observed. The rate of cell death approximated that observed when the cells were inoculated to ES solution at 0.5°C (Fig. 4) but was considerably less than that observed when the cells were incubated in oyster broth at 4°C.

Because cell concentration is known to affect survival during cold shock studies, this portion of the study was repeated with an inoculum of ca. 2 logs fewer cells. Results were consistent with those observed when higher cell concentrations were employed, and again the die-off rate approximated that seen when the cells were suspended in ES kept on ice.

For examining the possibility that the vibrios were penetrating into the oyster tissue and, therefore, were present in the mantle fluid in decreasing numbers, several oysters were homogenized after the 6-h sampling and examined immediately for numbers of *V. vulnificus* cells.

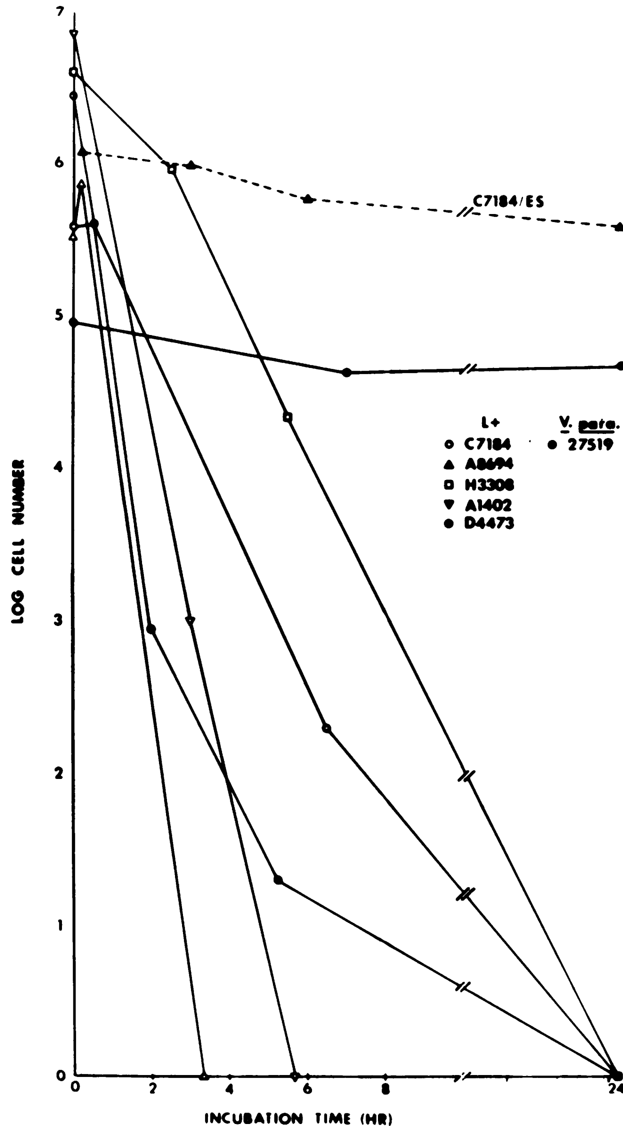

FIG. 1. *Survival of five V. vulnificus (L+) strains and of V. parahaemolyticus (V. para.) in homogenized oysters maintained at 4°C. Plating medium was BHI.*

Results indicated no significant increase in vibrios upon homogenizing, suggesting that active tissue penetration was not a factor in these studies.

DISCUSSION

The frequently lethal result of rapid chilling of bacterial cells has been termed cold shock and has been extensively studied. Numerous factors are known to affect the survival of bacteria during cold shock, including growth temperature (G. Houghtby and L. Liston, Bacteriol. Proc., p. 19, 1965) and phase of inoculum (2, 16, 18, 21), ionic strength and species of salts in the cell diluent (6, 16, 21), inoculum cell concentration (6, 7, 9, 21), chill rate of the test suspension (6, 16), incubation time at low temperature (21), plating medium (2, 7, 23), and the opportunity for the cells to repair the damage caused by the cold shock (4, 20, 25, 26). In general, cells which exhibit cold-induced death do so over a period of several days to weeks. *V. parahaemolyticus*, for example, has been reported to undergo only slight (0.05- to 0.8-log) reductions in viability when incubated for 24 h in fish and shellfish

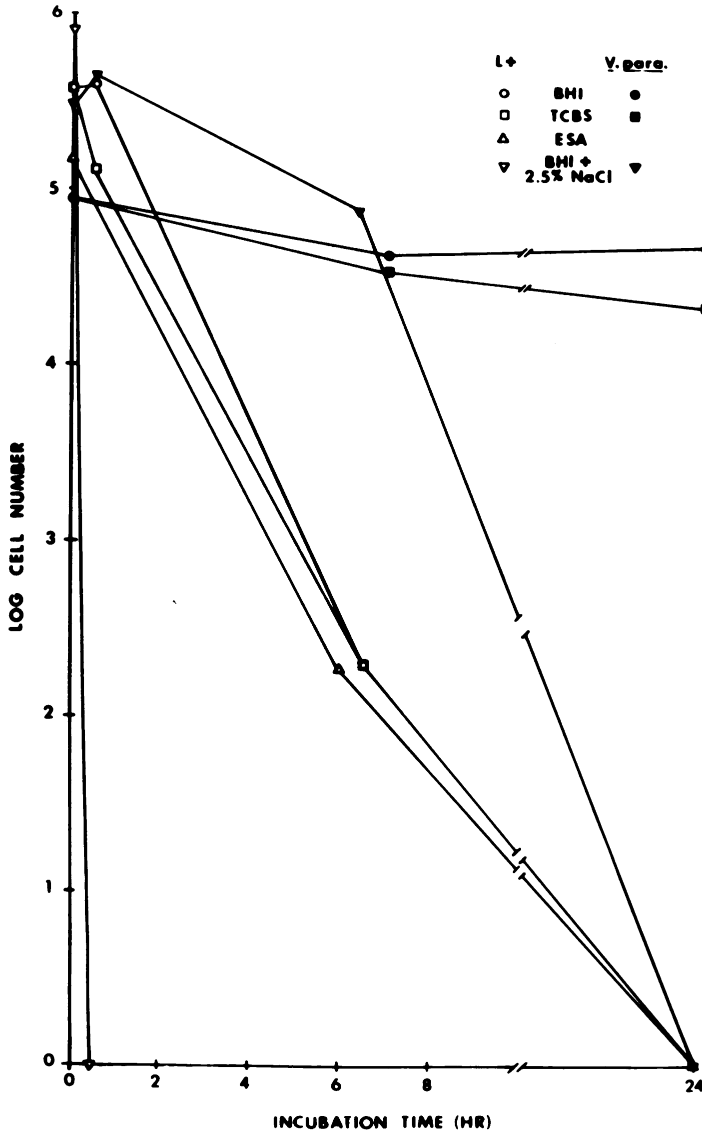

FIG. 2. *Effect of various plating media on the survival assays of V. vulnificus (L+) (strain C7184) and V. parahaemolyticus (V. para.) after incubation in oyster broth at 4°C. ESA, Estuarine salts agar.*

homogenates at temperatures between 0.6 and 3°C (14, 15, 23). Using *V. parahaemolyticus*-inoculated whole oysters, Goatcher et al. (7) reported that 3 to 5 days of incubation at 5°C were required to reduce the populations to nondetectable levels, and Johnson and Liston (10) found that *V. parahaemolyticus* could still be detected after 40 days of storage at 1°C on marine fish and shellfish. Similar findings were reported by Johnson et al. (11) and Vanderzant and Nickelson (24). Vanderzant and Nickelson (24) and Ma-Lin and Beuchat (14) have even reported increases in the *V. parahaemolyticus* population when cells were incubated at 3°C in shrimp or oyster homogenates, respectively.

Our studies with *V. parahaemolyticus* concur with these observations, with less than a 0.5-log reduction of *V. parahaemolyticus* observed in 24 h when cells were incubated in oyster homogenate at 4°C (Fig. 1). We observed a dramatic inactivation of *V. vulnificus* (unlike *V. parahaemolyticus*) when cells were incubated in oyster broth or on whole oysters at 0.5 to 4°C. The decline varied between strains and from experiment to experiment but was generally on the order of a 1-log reduction each 0.5 to 2 h. In one

TABLE 1. *Recovery of V. vulnificus cold shocked in oyster broth*

Time (h)	Plating medium	Total viable counts/ml of oyster broth	
		Unrepaired	Repaired[a]
0	BHI	3.8×10^5	
0.5	BHI	4.0×10^5	3.1×10^6
	BHI+S	3.3×10^5	1.3×10^6
	TCBS	1.3×10^5	1.6×10^5
6.5	BHI	2.0×10^2	$<10^2$[b]
	BHI+S	2.3×10^1	$<10^2$
	TCBS	2.0×10^2	$<10^2$
23.5	BHI	<10	$<10^2$
	BHI+S	<10	$<10^2$
	TCBS	<10	$<10^2$

[a] Repair results are given in cell numbers obtained after aliquots of the oyster broth held at 4°C for the times indicated were placed in BHI broth for 60 min at 37°C and then plated onto the indicated media.

[b] Minimum number detectable with this repair procedure.

strain (A1402), cells were undetectable after less than 4 h of incubation in oyster broth (Fig. 1). Only in the case of whole oysters inoculated with very high numbers (10^6 to 10^7) of *V. vulnificus* cells was any viability detectable at the 24-h sampling.

Dawe and Penrose (4), while studying the survival of coliforms in seawater, noted a high injury rate to these cells, but they also noted a high rate of survival. The ability of the cells to develop into colonies was dependent on the plating medium and on the ability of the cells to recover from the injury. A similar observation was made by Ray et al. (20), who examined the survival of cold-injured *V. parahaemolyticus* in seafood. These cells appeared to be nonviable unless allowed to repair themselves before plating. Studies reported here, examining the ability of *V. vulnificus* cells to repair the cold-induced damage, indicated that the cells were injured to a point of nonrecovery and were nonviable (Table 1).

As has been observed by previous investigators, the plating medium employed in cold shock studies is of special concern. It is generally felt that the low temperature induces membrane damage, leading to a greater sensitivity to a variety of compounds (5, 20, 21, 23). This damage is frequently manifested as a greater sensitivity to salt, so that the salt levels generally found in marine and estuarine media are lethal to stressed (cold-shocked) cells. This was found to be the case for both *V. parahaemolyticus* and *V. vulnificus* in the present study. Little difference in viability was observed for either organism when cells were plated on BHI or TCBS (these media contained 0.5 and 1.0% NaCl, respectively), or for *V. vulnificus* when ES agar was used (Fig. 2). The addition of salt to BHI (to a final concentration of 3%), however, produced a marked effect on both organisms, with *V. vulnificus* showing total lack of viability at the first (30-min) sampling and *V. parahaemolyticus* no longer detectable at 24 h. Both organisms require NaCl for growth (0.5% appears to be the minimum concentration required, and 1 to 3% appears to be the optimum) but not Mg^{2+} or K^+ (unpublished observations).

In an attempt to approximate a more realistic situation of *V. vulnificus*-contaminated oysters served on the half-shell in restaurants, we inoculated whole oysters kept on ice with various levels of the vibrio. When results for incubation of the cells on whole oysters were compared with those for incubation in oyster broth, a large difference in die-off rate was observed. Whereas cells incubated in the oyster homogenate showed a dramatic decrease in number (ca. 6 logs by 24 h) which greatly exceeded that seen when the cells were kept in ES at 4°C, cells inoculated onto iced whole oysters exhibited a slower decline in viability (ca. 3 logs in 24 h) which paralleled that of cells held in the iced diluent alone.

There appears, therefore, to be a lethal factor(s) released from the oysters during homogenizing. This factor is resistant to autoclaving and was absent in the mantle fluid. It appears to act selectively against *V. vulnificus*, because *V. parahaemolyticus* was insensitive to the oyster homogenate, and is not likely to be related to pH, because no appreciable differences in growth rates or yields of *V. vulnificus* cells at pH values of 6.3 to 8.2 have been observed in this laboratory (unpublished observations). Vanderzant and Nickelson (24) and Goatcher et al. (7) have also found pH not to be a factor in cell death in oysters following cold shock. Li (12) and Li et al. (13) have reported that aqueous extracts of ground oysters exhibit antibacterial activity, and it seems quite possible that one or more of these compounds is a factor in the die-off observed for *V. vulnificus*. The factor is not of consequence, however, unless the cells are chilled, because cells incubated in oyster broth at 25°C not only survive but increase in population density (Fig. 3).

The purpose of the present study was to examine the survival of *V. vulnificus* in raw oysters to provide insight into the role of oysters in the epidemiology of *V. vulnificus* infection. Such a role has been suggested by Blake et al. (3) based

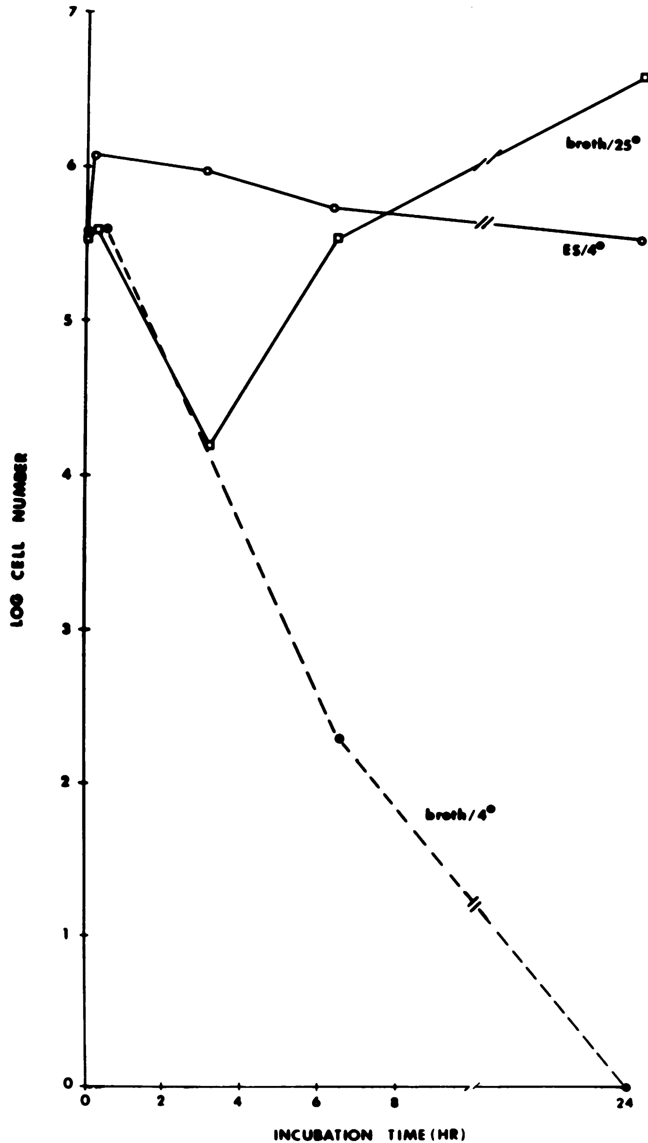

FIG. 3. *Effect of temperature on the antibiotic effect of the lethal factor released from homogenized oysters on V. vulnificus (strain C7184) and V. parahaemolyticus. Broth was oyster homogenate.*

on clinical histories. Data reported in the present study indicate that whole oysters kept on ice, unless contaminated with very high levels of *V. vulnificus* cells (ca. 10^6 to 10^7 cells per oyster), are not likely to transmit the bacterium. Further, there appears to be a heat-stable factor within oysters which selectively inactivates *V. vulnificus* cells (and not *V. parahaemolyticus* cells) when the bacterium is chilled.

Although homogenizing chilled shellfish is a standard method used for examining for bacterial contamination, the differences in *V. vulnificus* viability observed in this study with whole and homogenized oysters and the possibility of release of toxic compounds during the homogenizing process suggest that caution be exercised in using this procedure exclusively.

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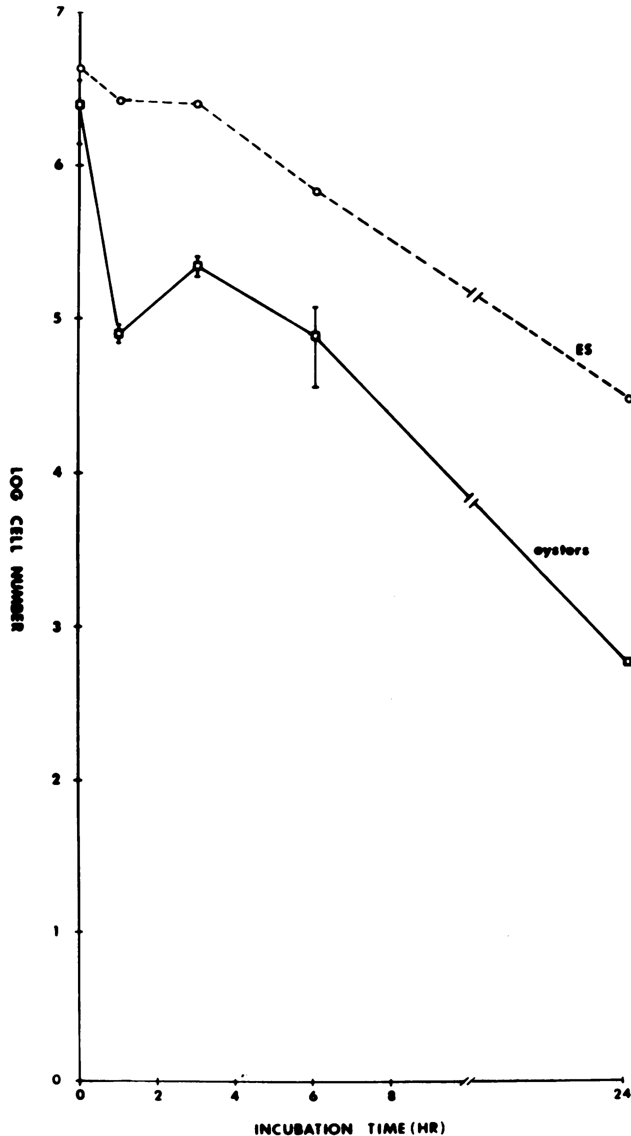

FIG. 4. *Survival of V. vulnificus (strain C7184) on whole oysters maintained at 0.5°C. Data points are the average of bacterial counts of two oysters sampled at each time. Vertical bars on data points represent individual counts.*

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