APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1992, p. 2771–2775
0099-2240/92/092771-05$02.00/0

Vol. 58, No. 9

Effects of Temperature Abuse on Survival of *Vibrio vulnificus* in Oysters

SUSAN K. MURPHY AND JAMES D. OLIVER*

Department of Biology, The University of North Carolina at Charlotte, Charlotte, North Carolina 28223

Received 20 April 1992/Accepted 10 June 1992

Opaque and translucent morphotypes of a Tn*phoA*-containing strain of *Vibrio vulnificus* were fed to oysters, which were subsequently stored at temperatures ranging from 0.5 to 22°C for 10 days. Samples of oysters were homogenized and plated at intervals to determine the cell density of *V. vulnificus* and total aerobic population of bacteria present. At all temperatures, the numbers of *V. vulnificus* (both morphotypes) declined over the 10-day study period. The same observation was made with a lower inoculum of *V. vulnificus*. Identical experiments with shucked oysters showed a more rapid decrease in *V. vulnificus*. Identical experiments with shucked oysters showed a more rapid decrease in *V. vulnificus* to levels below limits of detection. Little change in the total bacterial counts was observed in shellstock oysters at any of the test temperatures, whereas incubation at the higher temperatures (17 and 22°C) resulted in large increases in total counts in shucked oysters. These data suggest that temperature abuse of oysters may not be a factor in increasing the public health risk of *V. vulnificus* through raw oyster consumption. However, the data also suggest that even with proper storage, indigenous levels of *V. vulnificus* may remain sufficiently high in shellstock oysters to produce infection in compromised hosts.

Vibrio vulnificus is a pathogenic marine bacterium capable of producing severe infection in individuals with certain underlying chronic disease. Infections are generally associated with the consumption of raw seafood, particularly oysters, where this bacterium becomes concentrated through filter feeding. An unusual consequence of septicemia is the production of secondary skin lesions, particularly of the extremities, characterized by rapid swelling, erythema, edema, and necrosis of surrounding tissues within days. Approximately 60% of infected individuals die, although infections are limited primarily to persons with liver damage or other chronic disease resulting in elevated iron levels in serum (6).

In 1985, Yoshida et al. reported the existence of two colony morphotypes for *V. vulnificus* (11). They found a correlation between virulence and opacity and between virulence and the presence of capsular material. In 1987, workers in our laboratory found that only the opaque colony types were virulent, whereas the translucent isotypes were avirulent (9). Both opaque and translucent isotypes were used in this study in an attempt to distinguish variations, if any, between the two with regard to temperature abuse.

At all stages of harvest, transport, and storage, oysters may be exposed to temperature extremes. It is conceivable that, especially during elevated temperature abuse, *V. vulnificus* could increase in numbers within the oysters, creating a potentially greater health hazard for high-risk individuals who consume raw oysters. Such abuse could be responsible for the high incidence of infections associated with the oyster harvest areas along the Gulf coast of the United States.

The intent of this study was to simulate elevated temperature abuse that may occur in oysters and to compare the levels of *V. vulnificus* and total aerobic counts of bacteria in these oysters with those of oysters stored properly at refrigeration temperatures. Previous studies of this nature have required the use of enrichment cultures, most-probable-number enumeration procedures, or direct platings, all followed by taxonomic studies (1–3). In the present study, a transposon-containing strain of *V. vulnificus* that allowed direct plating and rapid (within 24 h) confirmation of the presence of *V. vulnificus* from oyster samples was used. This eliminated the tedious, time-consuming, and inherently inaccurate methods employed in previous studies.

MATERIALS AND METHODS

Bacterial strain. A transposon (Tn*phoA*)-containing strain of *V. vulnificus*, designated CVD713, was used throughout the study. Construction of such strains was described previously (10). Briefly, a stable, productive Tn*phoA* insertion was made into the chromosome; this insertion confers alkaline phosphatase activity and kanamycin resistance to the cells. To assess the effects of temperature abuse on strains of the opaque and translucent morphotypes, a spontaneous translucent mutant was isolated from the CVD 713 opaque strain and also employed in these studies. Both strains were maintained on L agar amended with 50 μg of kanamycin per ml.

Oysters. Oysters (*Crassostrea virginica*), ca. 8 to 10 g each including liquor, originated from the Chesapeake Bay area and were purchased from a local seafood distributor. Oysters were washed in clean, fresh tap water and placed in a 200-liter aquarium in a temperature-controlled room. Instant Ocean sea salts (Aquarium Systems) were used for the seawater source. The salinity of the water was adjusted to 20‰ at pH 8.0, and the temperature was maintained at 22°C. Tank water was aerated and filtered through a recirculating filter system at the rate of 830 liters per h. Oysters were acclimated to these conditions for 2 to 3 days before the studies were begun.

* Corresponding author.

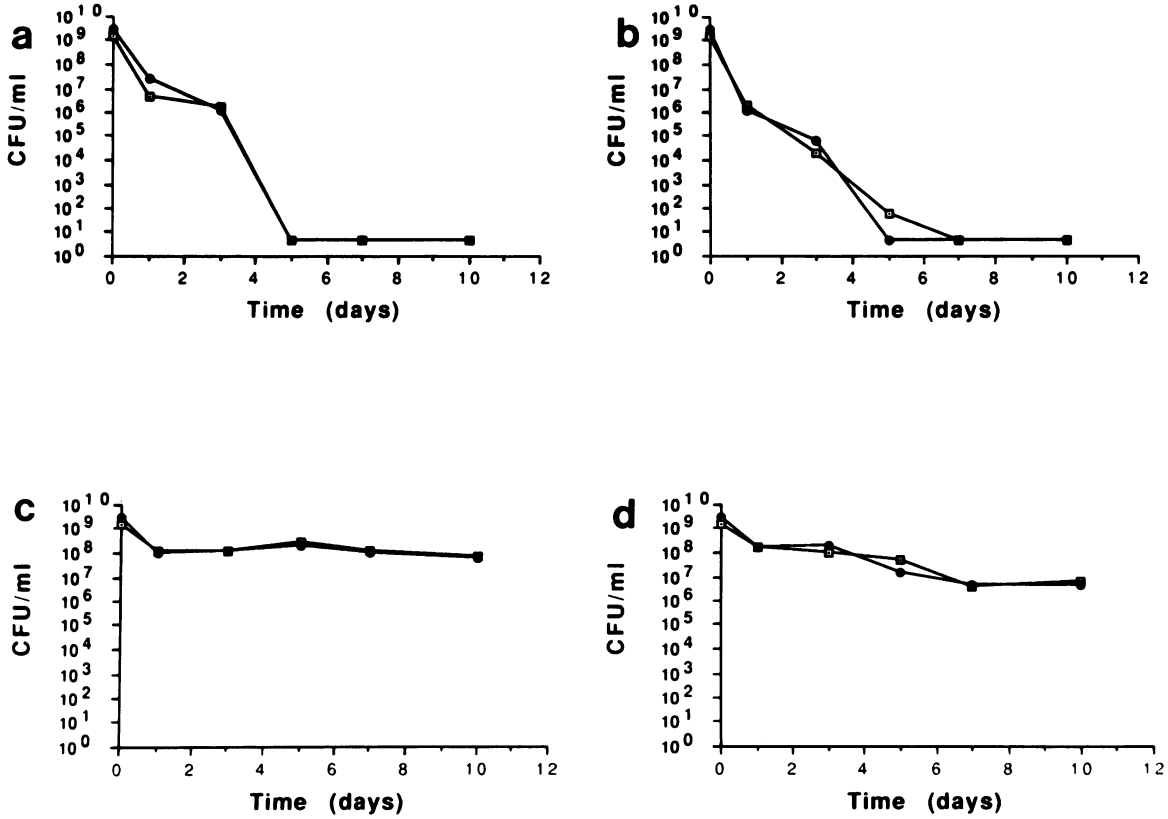

FIG. 1. Stability of the transposon Tn*phoA* in *V. vulnificus* CVD713 (opaque) incubated in artificial seawater for 10 days at temperatures from 0.5 to 24°C. Bacteria were plated onto L agar supplemented with glucose and BCIP (□) or the same medium with 50 μg of kanamycin per ml (●) to determine viability of the population and retention of the transposon. Oyster holding temperatures: 0.5°C (a), 5°C (b), 17°C (c), 24°C (d). Data for the 10°C sample (not shown) were the same for the two media.

Uptake of *V. vulnificus* by oysters. After acclimation, oysters were fed a mixture of 500 ml of algae (*Chlamydomonas* and *Chlorella* spp.) along with 15 ml of a standing overnight culture of CVD713 grown at 22°C in heart infusion broth (Difco) containing 50 μg of kanamycin per ml. Oysters typically were feeding within 15 min and were allowed to feed for 5 to 6 h. After the feeding, oysters were collected such that 10 oysters constituted a time zero sample. This sample was immediately analyzed upon removal from the aquarium to provide postfeeding baseline levels of *V. vulnificus*. The remainder of the oysters were divided into five groups consisting of at least 50 oysters each. These groups of oysters were placed on plastic trays and transferred to the experimental temperatures (0.5, 5, 10, 17, and 22°C) for subsequent testing during the 10-day study period.

Shucked oyster study. Oysters were purchased, washed, acclimated, and fed as described above. The opaque (virulent) strain of CVD713 was used for these experiments. After feeding, all oysters were aseptically shucked and placed in five sterile covered glass beakers at the five designated temperatures. At intervals, including time zero, oysters (n = 10) from each beaker, along with their liquor, were processed and plated to determine levels of *V. vulnificus* and total aerobic bacteria present.

Enumeration of bacteria. Samples were taken four times during the course of each experimental run. For the shellstock studies, 10 oysters were removed from the trays at each sampling time from each temperature. Oysters were aseptically shucked and weighed along with the liquor in each shell. For both the shellstock and shucked-oyster studies, an equal amount of artificial salt water was added to each sample, which was then blended for a total of 120 s with 15-s bursts at 5-s intervals. Serial dilutions of the homogenate were prepared in artificial salt water, and 0.1 ml aliquots were plated onto heart infusion agar (Difco) to monitor total aerobic counts and onto L agar amended with 50 mg of kanamycin, 2 g of glucose, and 40 mg of 5-bromo-4-chloro-3-indolylphosphate (BCIP; Sigma Chemical Co.) per liter of distilled water. BCIP is a chromagen that turns blue when hydrolyzed by the exported alkaline phosphatase mediated by the Tn*phoA* transposon in strain CVD713. This transposon also confers resistance to the kanamycin present in the medium. Glucose was added to repress the alkaline phosphatase activity naturally present in *V. vulnificus*. Plates were incubated for 24 h at 37°C; blue colonies appearing on the plates represented *V. vulnificus*. The results of the various studies were analyzed statistically with single degree-of-freedom contrasts among the means.

RESULTS

Transposon stability study. Tests were initially performed to determine the stability of the Tn*phoA* transposon insertion. The CVD713 opaque strain of *V. vulnificus* was cultured in heart infusion broth as described above. Cells were then suspended in artificial salt water and placed at the experimental temperatures (0.5, 5, 10, 17, and 24°C). At intervals of up to 12 days, aliquots of each suspension were

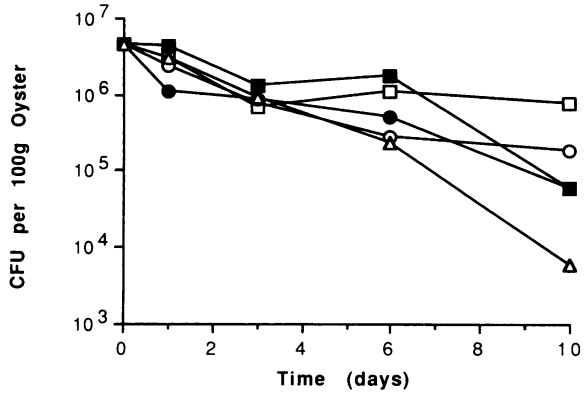

FIG. 2. Effect of temperature on survival of *V. vulnificus* CVD713 (opaque) in shellstock oysters. After uptake of the bacteria, oysters were transferred to various temperatures and held for up to 10 days. At intervals, oysters were removed, homogenized, and plated onto L agar supplemented with glucose, BCIP, and kanamycin (50 μg/ml) to determine the density of *V. vulnificus* CVD713. Oyster holding temperatures were 0.5°C (○), 5°C (□), 10°C (●), 17°C (■), and 22°C (△).

plated onto L agar amended with glucose and BCIP with and without the selective pressure of the kanamycin. At 0.5 and 5°C, the plate counts of *V. vulnificus* underwent a rapid decline, which is known to be a result of this bacterium's entering into the viable but nonculturable state (8). The transposon, however, was stable at all temperatures over the 10-day sample period (Fig. 1). Further, we have found that this insertion does not affect putative virulence factors of *V. vulnificus* (unpublished results). Additionally, CVD713 was examined for its response to starvation, which has been described for *V. vulnificus* (8). In all tests, strain CVD713 was similar to the parent strain.

Shellstock oysters, CVD713 (opaque), large inoculum. At all temperatures, levels of the opaque morphotype of *V. vulnificus* declined over the 10-day study period (Fig. 2). At 5°C, there was roughly a 0.5-log-unit decrease, whereas at 0.5, 10, and 17°C there was a 1- to 2-log-unit decrease. The most drastic decrease in *V. vulnificus* numbers was at 22°C; the number of CFU declined from 4.7×10^6 to 6.0×10^3/100 g, a decrease of almost 3 orders of magnitude.

Total aerobic counts of bacteria present in the oysters, as monitored on heart infusion agar, showed small but significant ($P < 0.01$) decreases over the sampling period at all temperatures except 17°C (Fig. 3). The time zero sample contained 4.0×10^7 CFU/100 g; a slight but significant increase (to 1.8×10^8 CFU/100 g) was observed only for the 17°C sample.

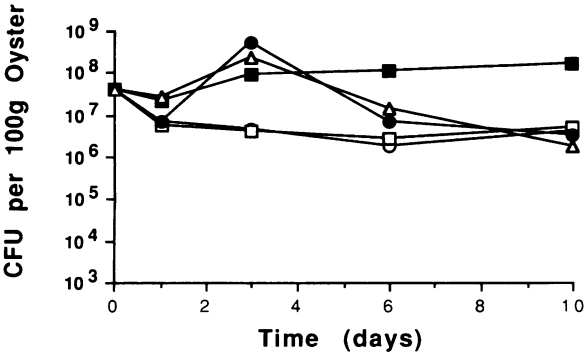

FIG. 3. Effect of temperature on survival of aerobic bacteria in shellstock oysters. Oysters were held at various temperatures for up to 10 days, with samples plated at intervals onto heart infusion agar. Symbols are as described in the legend to Fig. 2.

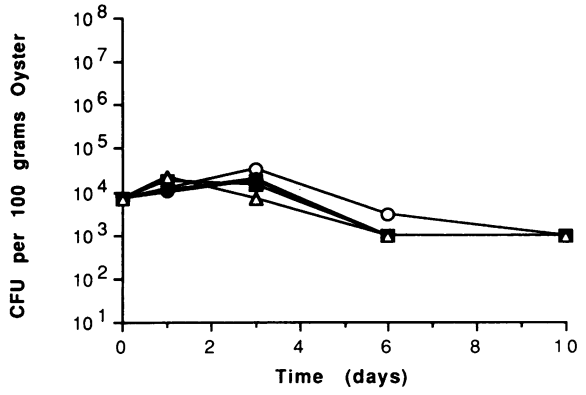

FIG. 4. Survival of small inocula of *V. vulnificus* CVD713 (opaque) in shellstock oysters. The study shown in Fig. 2 was repeated, but the inoculum was modified to provide a starting *V. vulnificus* density of ca. 10^4 CFU/100 g of oyster. Symbols are as described in the legend to Fig. 2.

Shellstock oysters, CVD713 (opaque), small inoculum. The time zero level of *V. vulnificus* in these studies was 7.0×10^3 CFU/100 g (Fig. 4). By day 3, *V. vulnificus* had increased slightly at all temperatures, to roughly 10^4 CFU/100 g. By day 10, however, culturable *V. vulnificus* had declined to at or below the limits of detection of our plating technique ($<1.0 \times 10^3$ CFU/100 g).

The total aerobic bacterial counts remained relatively stable at all but the highest and lowest temperatures over the 10-day study period (Fig. 5). Only at 0.5 and 22°C were significant ($P < 0.01$) declines observed.

Shellstock oysters, CVD713 (translucent), large inoculum. Beginning with a time zero cell density of 1.3×10^7 CFU/100 g, a gradual decline (ca. 3 log units) of the translucent strain of *V. vulnificus* occurred over the 10 days at all temperatures (Fig. 6).

The total aerobic bacterial count at time zero was 6.2×10^7 CFU/100 g. A significant ($P < 0.01$) increase in the count

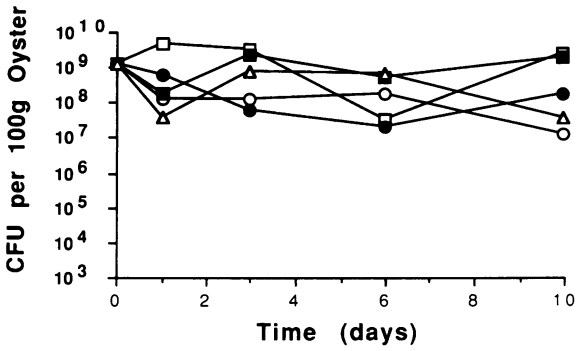

FIG. 5. Effect of temperature on survival of aerobic bacteria in shellstock oysters that received small inocula of *V. vulnificus* cells. See the legend to Fig. 3 for details.

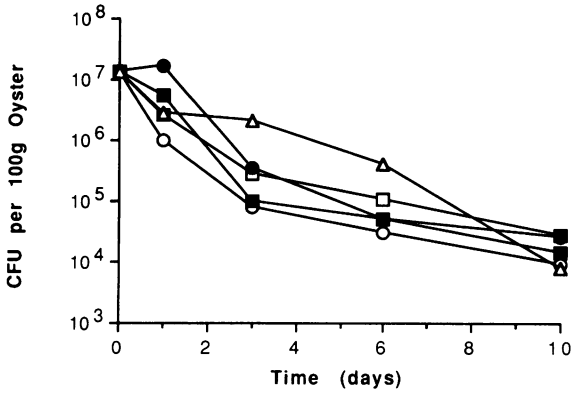

FIG. 6. Survival of *V. vulnificus* CVD713 (translucent strain) in shellstock oysters. The study shown in Fig. 2 was repeated, and the translucent colony morphotype of *V. vulnificus* was used. See the legend to Fig. 2 for details.

(to 2.6×10^{10} CFU/100 g) was observed only for the 22°C samples after 10 days (Fig. 7).

Shucked oysters, CVD713 (opaque). By day 2 of this study, there was a 3-log-unit decrease in *V. vulnificus* numbers at 0.5, 5, and 10°C (Fig. 8). By day 5, the 17 and 22°C samples also had dropped over 3 log units. Oyster meats at 22 and 17°C were discarded at days 8 and 12, respectively, because of putrefaction. In samples of oyster meat kept at these two temperatures before they were discarded, *V. vulnificus* was below the levels of detection (10^3 CFU/100 g). By day 12, the 0.5, 5, and 10°C samples were at or below detectable limits for *V. vulnificus* as well.

The total aerobic counts of these oyster meats showed an opposite trend (Fig. 9). Beginning at 5.4×10^7 CFU/100 g at time zero, large increases (to 1.6×10^{11} CFU/100 g) were evident by day 5 in the 22°C sample. The 17°C oysters also increased to 6.7×10^{10} CFU/100 g by day 8, whereas the counts for samples at 0.5, 5, and 10°C remained relatively stable.

DISCUSSION

Throughout these studies, one surprising but obvious consistency was that elevated temperature abuse in oysters, both shellstock and shucked meats, did not contribute to an increase in *V. vulnificus* levels (Fig. 2, 4, 6, and 8). These findings are in agreement with an earlier report by Kaysner et al. (3), who described decreases in *V. vulnificus* cell numbers in shellstock and shucked oysters at temperatures of up to 22°C. Similarly, they found that *V. vulnificus* remained at culturable levels in the large-inoculum experiments at all temperatures over the 10-day course (Fig. 2 in ref. 3). Whereas the *V. vulnificus* levels in shucked oysters in both the Kaysner et al. study and ours declined, Kaysner et al. (3) ended their study at 6 days and found a less drastic decrease (roughly 1 log unit) in *V. vulnificus* cell numbers at 4°C. By comparison, in our study, the levels of *V. vulnificus* had declined by 3 or more log units by day 5 and were at or below the detection limit by day 12. The discrepancy in the rapidity with which *V. vulnificus* decreased in these studies may be attributable to the fact that in the study by Kaysner et al. (3), oysters were injected into the gut area after the oysters were shucked, whereas in the present study the oysters obtained *V. vulnificus* through natural filter feeding and then were shucked. Our study more closely represents what would occur in nature. There was also a major difference in the method by which *V. vulnificus* was assayed in the Kaysner et al. (3) study. They employed alkaline peptone water enrichment steps with direct platings to cellobiose-polymyxin B-colistin agar (5) and sodium dodecyl sulfate-agar (4).

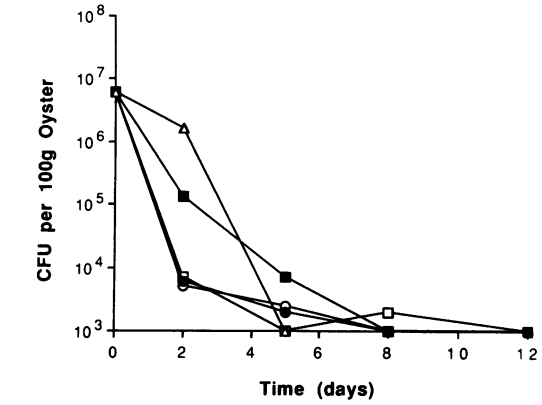

FIG. 8. Survival of *V. vulnificus* CVD713 (opaque) in shucked oysters. After uptake of *V. vulnificus*, oysters were shucked and placed at the various incubation temperatures for up to 12 days. Oysters were then examined for *V. vulnificus* as described in the legend to Fig. 2.

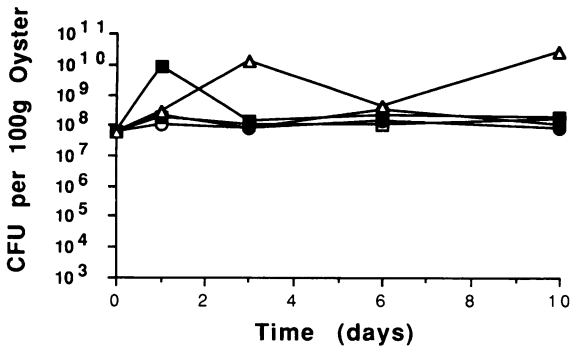

FIG. 7. Effect of temperature on survival of aerobic bacteria in shellstock oysters receiving the translucent colony morphotype of *V. vulnificus*. See the legend to Fig. 3 for details.

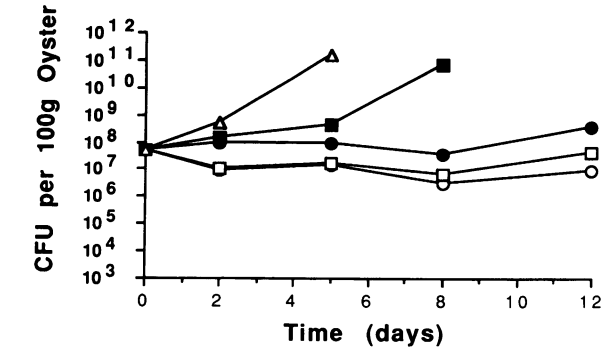

FIG. 9. Effect of temperature on survival of aerobic bacteria in shucked oysters. After storage of the oysters as described in the legend to Fig. 8, the oysters were examined for total aerobic bacteria. See the legend to Fig. 3 for details.

It is interesting that there was a rapid decrease in plate counts exhibited by *V. vulnificus* incubated in artificial salt water at 0.5 or 5°C (Fig. 1) compared with the relative stability of the population in oysters at these temperatures (Fig. 2, 4, and 6). Apparently, *V. vulnificus* does not enter into the viable but nonculturable state (8) in oysters, or it requires more than the 10-day period employed in these studies. These possibilities are currently under investigation in our laboratory.

Altering the initial inoculum concentration (Fig. 4) did not significantly affect our results. The smaller-inoculum studies were performed to eliminate the possibility that the high cell density of *V. vulnificus* present in our experiments was at a level that would prevent an increase in number after temperature abuse. It was conclusively shown that this was not the case; the shellstock oysters feeding on the smaller inoculum also showed a decrease in *V. vulnificus* over the 10-day storage period.

The translucent isotype of this strain responded similarly (Fig. 6); the *V. vulnificus* cell numbers decreased over the course of the study. Although it is not known to what degree the translucent variety of this bacterium exists in nature, it is known that conversions between the opaque and translucent isotypes can occur (9). Experiments performed in this laboratory showed no conversions between the translucent and opaque morphotypes.

Studies by Hood et al. (2) found increases in *V. vulnificus* numbers in shellstock oysters at 8 and 20°C after 7 days, with decreases evident only at 14 and 21 days. In the present study, decreases were evident in *V. vulnificus* levels at all temperatures after 10 days of storage. Another study by Cook and Ruple (1) reported that within an 18-h period from time of harvest to arrival at the processing plant, *V. vulnificus* levels in shellstock oysters had increased by up to 3 orders of magnitude. Additionally, at 22 and 30°C, *V. vulnificus* levels increased by 1 log unit after 1 day of storage in shellstock oysters (1). Cook and Ruple concluded that transport temperatures were a controlling factor in determining levels of *V. vulnificus* in oysters.

The reports of Hood et al. (2) and Cook and Ruple (1) do not agree with our findings, perhaps because of the enumeration methods employed in their studies (most-probable-number techniques, alkaline peptone enrichment cultures, taxonomic studies, etc.) to isolate and identify *V. vulnificus*. These steps may have led to an inaccurate portrayal of the presence of this bacterium in oysters. Indeed, the proper identification of this bacterium from natural sources is extremely difficult (6). Although standard taxonomic tests can be used to this end, the use of gene probes and/or monoclonal antibodies has distinct advantages in correctly identifying this important pathogen (7). Similarly, the use of the Tn*phoA* strain in the present study eliminated these tedious, time-consuming, and often inaccurate enumeration procedures and allowed direct and specific results within 1 day.

Although it was clear in our study that levels of *V. vulnificus* did not increase during storage at elevated temperatures, the same was not always true for other indigenous species of aerobic bacteria in the oysters. Whereas at most temperatures shellstock bacteria showed little change over the 10-day incubation period, increases in total aerobic counts of bacteria were evident at the highest storage temperature (Fig. 7), indicating that it is possible for flora in oysters to multiply during temperature abuse. Such a temperature response was especially evident in the shucked oyster study, in which the aerobic counts showed great increases at both 17 and 22°C. This response is probably due to the lack of any host defenses in the dead oysters.

We conclude that, although a higher storage temperature does not, in itself, cause *V. vulnificus* to multiply within the oyster and thus increase the possibility of disease outbreak, proper storage temperatures are by no means enough to ensure that consumption of *V. vulnificus* is without some potential public health hazard.

ACKNOWLEDGMENTS

We thank the laboratory of J. Glenn Morris, Jr., for supplying the CVD713 *V. vulnificus* strain used in these experiments.

This study was supported by North Carolina Sea Grant R/MER-20.

REFERENCES

1. **Cook, D. W., and A. Ruple.** 1989. Indicator bacteria and *Vibrionaceae* multiplication in post-harvest shellstock oysters. J. Food Prot. **52**:343–349.
2. **Hood, M. A., G. Ness, G. Rodrick, and N. Blake.** 1983. Effects of storage on microbial loads of two commercially important shellfish species, *Crassostrea virginica* and *Mercenaria campechiensis*. Appl. Environ. Microbiol. **45**:1221–1228.
3. **Kaysner, C. A., M. Tamplin, M. Wekell, R. Stott, and K. Colburn.** 1989. Survival of *Vibrio vulnificus* in shellstock and shucked oysters (*Crassostrea gigas* and *Crassostrea virginica*) and effects of isolation medium on recovery. Appl. Environ. Microbiol. **55**:3072–3079.
4. **Kitaura, T., S. Doke, I. Azuma, M. Imaida, K. Miyano, K. Hadura, and E. Yabuuchi.** 1983. Halo production by sulfatase activity in *V. vulnificus* and *V. cholerae* O1 on a new selective sodium dodecyl sulfate-containing medium: a screening marker in environmental surveillance. FEMS Microbiol. Lett. **17**:205–209.
5. **Massad, G., and J. Oliver.** 1987. New selective and differential medium for *Vibrio cholerae* and *Vibrio vulnificus*. Appl. Environ. Microbiol. **53**:2262–2264.
6. **Oliver, J. D.** 1989. *Vibrio vulnificus*, p. 569–599. *In* M. Doyle (ed.), Foodborne bacterial pathogens. Marcel Dekker, Inc., New York.
7. **Oliver, J. D., K. Guthrie, J. Preyer, A. Wright, L. Simpson, R. Siebeling, and J. G. Morris, Jr.** 1992. Use of colistin-polymyxin B-cellobiose agar for isolation of *Vibrio vulnificus* from the environment. Appl. Environ. Microbiol. **58**:737–739.
8. **Oliver, J. D., L. Nilsson, and S. Kjelleberg.** 1991. Formation of nonculturable *Vibrio vulnificus* cells and its relationship to the starvation state. Appl. Environ. Microbiol. **57**:2640–2644.
9. **Simpson, L. M., V. White, S. Zane, and J. Oliver.** 1987. Correlation between virulence and colony morphology in *Vibrio vulnificus*. Infect. Immun. **55**:269–272.
10. **Wright, A. C., L. Simpson, J. Oliver, and J. G. Morris, Jr.** 1990. Phenotypic evaluation of acapsular transposon mutants of *Vibrio vulnificus*. Infect. Immun. **58**:1769–1773.
11. **Yoshida, S.-I., M. Ogawa, and Y. Mizuguchi.** 1985. Relation of capsular materials and colony opacity to virulence of *Vibrio vulnificus*. Infect. Immun. **47**:446–451.