APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1995, p. 2624–2630 Vol. 61, No. 7
0099-2240/95/$04.00+0

Entry into, and Resuscitation from, the Viable but Nonculturable State by *Vibrio vulnificus* in an Estuarine Environment

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Received 7 November 1994/Accepted 9 May 1995

Using plate counts, total cell counts, and direct viable counts, we examined the fate of cells of *Vibrio vulnificus* placed into natural estuarine waters during both winter and summer months. Cells inoculated into membrane diffusion chambers and placed into estuarine waters entered into a viable but nonculturable (VBNC) state in January and February, when the water temperatures were low (average, <15°C). In contrast, when cells in the VBNC state were placed into the same waters in the warmer months of August through November (average water temperature of ca. 21°C), the cells appeared to undergo a rapid (typically, within 24 h) resuscitation to the fully culturable state. These results were independent of whether the cells were in the logarithmic or stationary phase and whether they were encapsulated or not. This study indicates that the inability to isolate *V. vulnificus* from cold estuarine sites may be accounted for by entrance of the cells into a VBNC state and that recovery from this state in natural environments may result from a temperature upshift.

Vibrio vulnificus is an estuarine bacterium which is capable of causing life-threatening infections in humans following either ingestion (typically, of raw oysters) or contamination of a wound (13). The bacterium is readily isolated from estuarine waters and filter-feeding molluscan shellfish, especially in summer months, when the incidence of infection with this bacterium is highest. However, isolation of the bacterium from these same sites during winter months and from naturally cold estuarine waters is difficult (13). While it is possible that *V. vulnificus* is able to "overwinter" in the intestines of estuarine fish, as has been suggested by DePaola et al. (1a), it is also possible that the inability to culture *V. vulnificus*, and many other estuarine vibrios, from cold waters is due, at least in part, to the entrance of these bacteria into a viable but nonculturable (VBNC) state (14). In this state, the cells can be demonstrated to remain viable but no longer grow in or develop colonies on the nonselective media normally employed for their culture. At least 12 genera of bacteria have been shown to enter this state (14), which may serve as a survival strategy against adverse environmental conditions. In *V. vulnificus*, the VBNC state is induced by a temperature downshift (24), and resuscitation from this state has been reported following removal of the temperature stress (12).

Most of the reports which have investigated the VBNC state (14), including those on *V. vulnificus* (6, 12, 16, 17, 23), have employed laboratory microcosms. While the data obtained from such studies are consistent with the assumption that the inability to isolate *V. vulnificus* from cold estuarine waters is due to entry of these cells into a VBNC state and that resuscitation from this state when the waters become warm would account for their renewed culturability, this has not been demonstrated in a natural environment. One approach to such studies is the use of the membrane diffusion chambers developed by McFeters and Stuart (7). These units retain the bacterial cells within a plexiglass chamber, but the membranes which form the barrier to the external environment allow the cells to experience external temperature and salinity changes and to respond to a continuous diffusion of nutrients and other molecules into and out of the chambers. Such chambers have been used extensively by McFeters and coworkers, as well as others (reviewed in reference 7).

In the present study, to determine if *V. vulnificus* is able to enter a VBNC state in natural environments, we inoculated fully culturable cells of *V. vulnificus* into membrane diffusion chambers and placed the chambers into estuarine waters during winter months. To monitor this process, we determined the total counts and direct viable counts (DVCs), as well as the plate counts, of the cells in these chambers. To demonstrate whether resuscitation from the VBNC state was possible when the cells were incubated in warm waters, we used the same protocol but with an inoculum of cells which were in the VBNC state. This is the first such study undertaken for any bacterium, the results of which suggest that the VBNC state is a survival strategy similar to that which follows nutrient starvation for non-spore-forming cells in a natural environment (4, 9, 19).

MATERIALS AND METHODS

Bacteria and culture conditions. *V. vulnificus* CVD713, carrying a transposon (Tn*phoA*) imparting resistance to kanamycin and production of alkaline phosphatase (25), was employed in these studies. Both the opaque (encapsulated) form and a translucent (nonencapsulated) isogenic spontaneous mutant were used (21). Cells were grown to the early logarithmic phase (optical density at 610 nm of 0.13 to 0.18) or to the stationary phase (overnight growth) in a peptone-yeast extract marine medium (MSWYE broth; reference 12) at room temperature (ca. 22°C).

Preparation of microcosms and membrane diffusion chambers. For studies examining the entry of cells into a VBNC state in the environment, cells grown as described above were diluted 100-fold in artificial seawater (ASW; reference 24) and placed directly into membrane diffusion chambers (see below). For studies examining resuscitation of nonculturable cells, microcosms containing diluted cells were first subjected to a temperature downshift to 5°C in the laboratory to induce a VBNC state. Cells were considered to have entered a VBNC state when $<10^1$ CFU/ml could be detected in the population.

To examine entry into, and exit from, the VBNC state in estuarine waters, aliquots of diluted microcosm cell suspensions, either culturable or nonculturable, were added to fill 25-ml sterile chambers (7, 20) to which 76-mm-diameter, 0.2-μm-pore-size polycarbonate filters (Poretics Corp.) had been affixed. Experimental chambers were then placed ca. 20 cm below the surface in estuarine waters located at Wrightsville Beach, N.C.

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Portions of the same VBNC populations used for these in situ studies were also placed at room temperature (ca. 21°C) in the laboratory to monitor resuscitation, while others were maintained in the laboratory at 5°C as controls for those studies examining induction of a VBNC state in the natural environment. In our initial laboratory studies, laboratory resuscitation was attempted in disposable screw-cap centrifuge tubes. Subsequent studies examined resuscitation by cells maintained in membrane diffusion chambers suspended in sterile ASW.

Bacterial media and viability assays. The use of a medium (Tn agar) containing kanamycin and the colorimetric alkaline phosphatase substrate 5-bromo-4-chloro-3-indolylphosphate (BCIP) permitted specific detection and enumeration of culturable cells of strain CVD713 (2, 11), which produces bright blue colonies on this medium. The specificity of this medium for strain CVD713 was further indicated by the fact that no blue colonies were detected when estuarine water from the test site was plated onto this medium. In some cases, up to 200 ml of the sample was filtered through a 0.2-μm-pore-size filter and the filter was plated to determine culturability, increasing the sensitivity of the plate counts to as few as <0.005 CFU/ml. As described in our previous studies (6, 12, 24), total acridine orange direct cell (AODC) count and DVC assays done by the method of Kogure et al. (5) were employed to demonstrate that the cells had entered into a VBNC state.

Monitoring of environmental conditions. The studies described here for cells entering the VBNC state were carried out between January and March, whereas the studies on resuscitation of cells from the VBNC state were conducted between July and November. Salinities and temperatures of the seawater adjacent to the environmental chambers were monitored with a portable salinometer-temperature meter (YSI Instruments).

RESULTS

Studies on entry into the VBNC state. In our first studies examining the entry of cells into the VBNC state, either log- or stationary-phase cells of *V. vulnificus* (both the opaque and translucent morphotypes) were placed into estuarine waters which, during January and February, exhibited temperatures of 10 to 15°C (median of 10°C) and salinities of 10 to 19.5 ppt (average of 14.5 ppt). As shown in Fig. 1 and 2, both morphotypes, whether in the stationary (Fig. 1) or log (Fig. 2) phase, entered a VBNC state within 14 to 20 days. On the basis of the slope of the plate count curves, however, it is likely that these cells actually became nonculturable much earlier (within 5 to 7 days) than is indicated with these sample intervals. Throughout this period, total direct cell counts in both studies remained near the original inoculum level of 10^6 to 10^7 cells/ml, with DVC populations of 10^4 to 10^5 cells/ml remaining in the January study (Fig. 1) and 10^5 to 10^6 cells/ml remaining in the February study (Fig. 2).

In a subsequent study carried out in early March, when water temperatures were ca. 10 to 18°C (median of 15°C) and salinities were 12 to 20 ppt (average, 15.5), both log- and stationary-phase cells were examined. In this study (Fig. 3), as in the earlier studies, both log- and stationary-phase cells, regardless of colony morphotype, decreased ca. 4 log CFU in 7 days. Despite this rapid loss of culturability, total direct cell counts indicated little decrease in the total population density while DVCs revealed that a significant viable population was maintained (Fig. 4). This was true regardless of the morphotype of the cells and whether the cells were in the logarithmic (Fig. 4a) or stationary (Fig. 4b) phase prior to insertion into the natural water.

Control cells maintained at 5°C in the laboratory (Fig. 5) rapidly lost culturability (3 to 5 logs in 7 days), nearly paralleling the decline observed in the experimental cells present in the estuarine waters. AODC and DVC data indicated that these control cells were in a VBNC state (data not shown).

Resuscitation studies. To examine whether nonculturable cells of *V. vulnificus* could exit the VBNC state in an estuarine environment, cells induced to enter a VBNC state in the laboratory were placed into chambers at the same estuarine site during warm-water months and monitored for appearance of culturable CVD713 cells.

As shown in Fig. 6, cells of both the opaque and translucent morphotypes examined in the first study became nonculturable ($<1 \times 10^1$ CFU/ml) within 15 days when incubated in the laboratory at 5°C in ASW. These cells were placed into estuarine waters 7 days after becoming nonculturable. Initial water conditions for this September-October study included a temperature of 22°C and a salinity of 26 ppt. These values fluctuated between 22 and 24°C and between 19 ppt (due to significant rain on one occasion) and 30 ppt during the first 4 days the chambers were present in the water.

Cells of the translucent morphotype appeared to resuscitate and become fully culturable (reached the time zero AODC levels) within the first day of estuarine water incubation (Fig. 6). The opaque cell population became partially culturable (5×10^3 CFU/ml) during the first day in situ and fully culturable

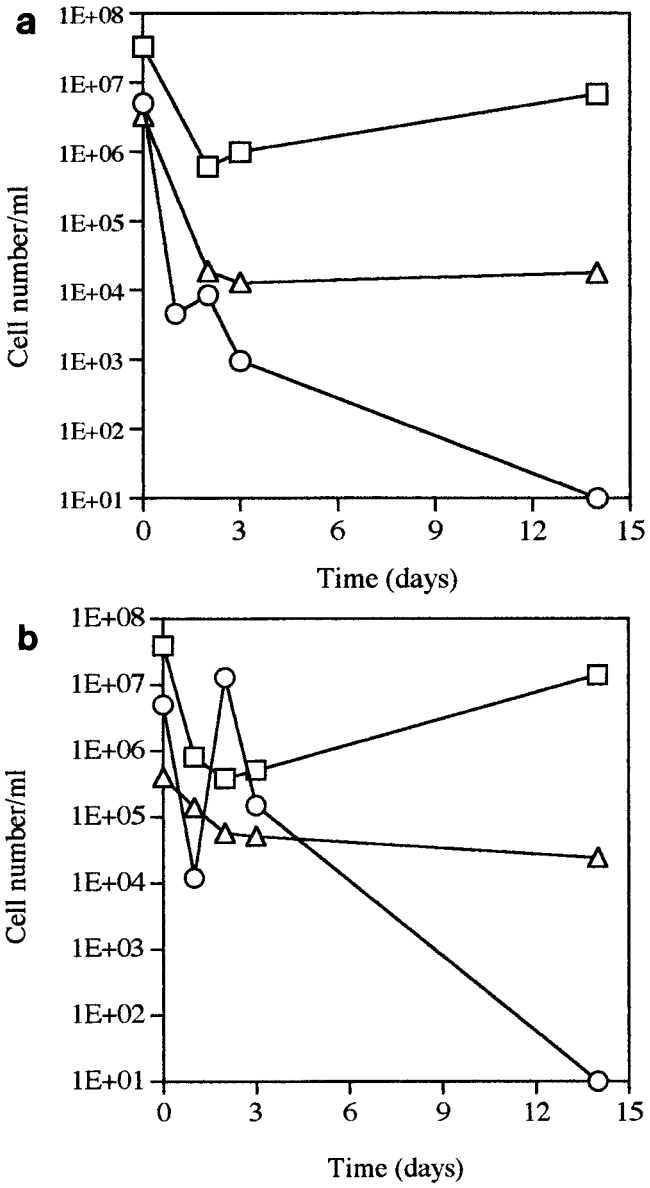

FIG. 1. Entrance of stationary-phase cells of both the opaque and translucent morphotypes of *V. vulnificus* into a VBNC state in 10 to 15°C estuarine waters. Shown are results of plate count (○), total direct count (□), and DVC (△) assays for each strain. Panels: a, opaque morphotype; b, translucent morphotype.

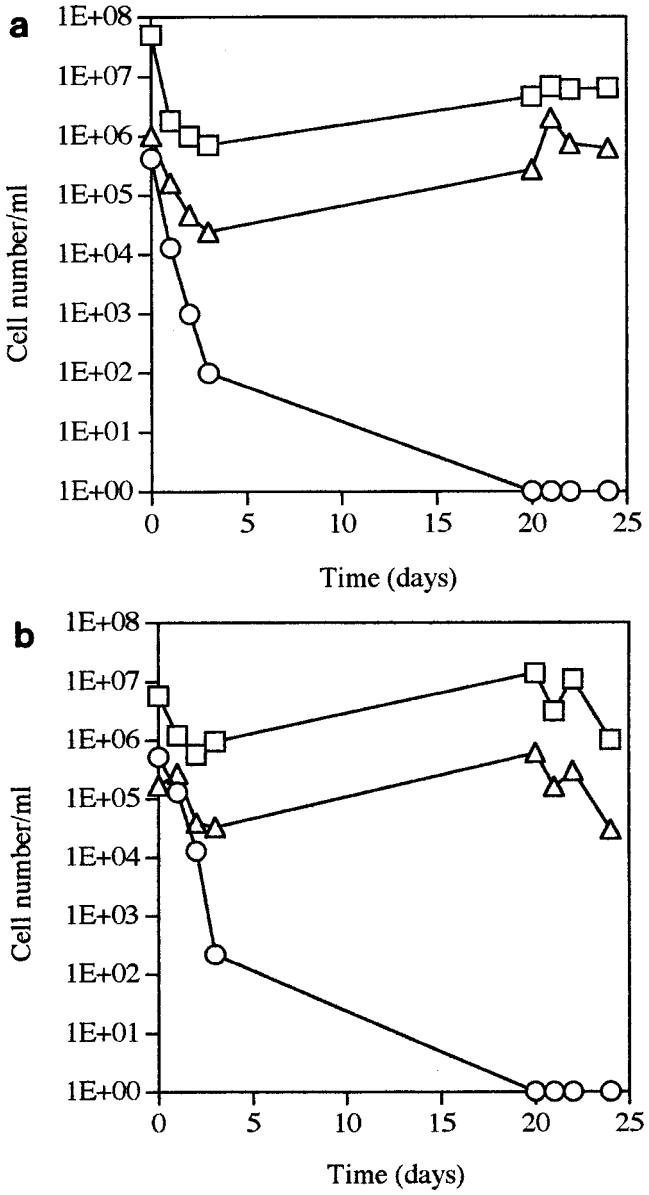

FIG. 2. Entrance of logarithmic-phase cells of both the opaque and translucent morphotypes of *V. vulnificus* into a VBNC state in 10 to 15°C estuarine waters. Shown are results of plate count (○), total direct count (□), and DVC (△) assays for each strain. Panels: a, opaque morphotype; b, translucent morphotype.

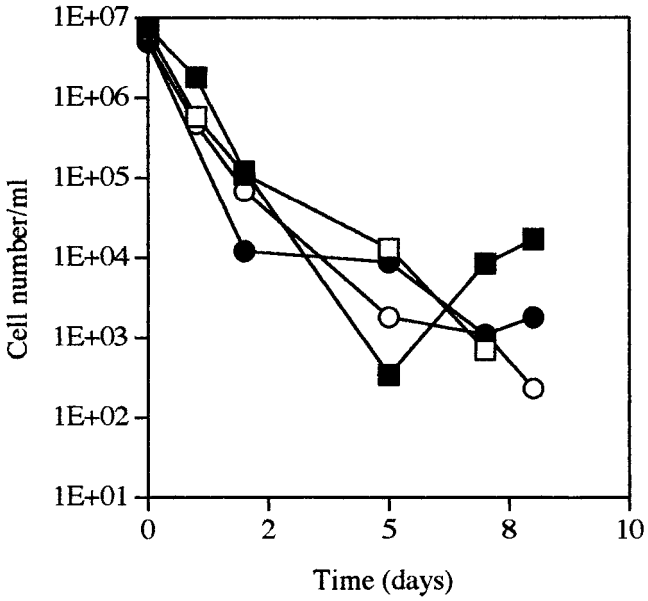

FIG. 3. Entrance of logarithmic- and stationary-phase cells of both the opaque and translucent morphotypes of *V. vulnificus* into a VBNC state in 10 to 18°C estuarine waters. Shown are results of plate counts for stationary-phase (squares) and logarithmic-phase (circles) cells of both the opaque (closed symbols) and translucent (open symbols) morphotypes.

within 4 days (no samples were taken on days 2 and 3). With both morphotypes, the cell densities which resulted approximated those (ca. 10^8 CFU/ml) originally inoculated into the chambers.

A repeat of the September-October study was performed in November, at which time the water temperatures were 16 to 19°C and salinities were fairly constant at 26 to 27 ppt. Log-phase cells of both the opaque and translucent morphotypes rapidly entered a VBNC state (<10 CFU/ml) when incubated in the laboratory in ASW at 5°C (Fig. 7). These cells were placed into chambers at the same estuarine site 4 days after they had become nonculturable. Similar to the results obtained in the September-October study, both the opaque and translucent cells demonstrated apparent resuscitation (to 6×10^6 and 1×10^7 CFU/ml, respectively) by the first sampling (24 h). By the next sample time (4 days later), both populations had reached ca. 10^8 CFU/ml.

The following year, two more resuscitation studies were performed at the same estuarine site. In each of these studies, however, the level of culturability of the cells placed into the chambers was <0.04 CFU/ml. This was achieved through the filtration and plating of a minimum microcosm sample size of 25 ml. As a result, the possibility that the increase in cell number observed in the first two resuscitation studies was due to growth of a few residual cultural cells could be all but eliminated. Further, and unlike the earlier resuscitation studies, DVC (2.7×10^4 to 3.1×10^5 cells/ml) and AODC (3.5×10^5 to 8.6×10^5 cells/ml) data were known for the cell populations which were placed into the chambers.

In the first of these studies (carried out in August), water temperatures varied from a low of 17°C to a high of 36°C (average of 25°C). In this case, both opaque and translucent cells which had been in a VBNC state for 6 days demonstrated partial resuscitation (to 10^2 to 10^3 CFU/ml) by the first sampling (12 h) and reached their respective original population DVC levels (2.7×10^4 and 7.2×10^4 cells/ml, respectively) by 60 h (Fig. 8).

That the increase observed in strain CVD713 plate counts in the study shown in Fig. 8 was due to resuscitation of cells from the VBNC state and not to growth of culturable cells undetected during our assays is suggested by the results obtained when fully culturable cells were studied. As shown in Fig. 9, culturable cells diluted to ca. 1 CFU/ml prior to being placed into the estuarine water were able to attain a culturable population of only 5×10^2 CFU/ml, considerable less than that (10^4 to 10^5 CFU/ml) seen in the companion study (Fig. 8) with nonculturable cells.

At the same time that the VBNC cell populations of this study were placed into estuarine waters, portions of the same VBNC populations (opaque and translucent) were transferred

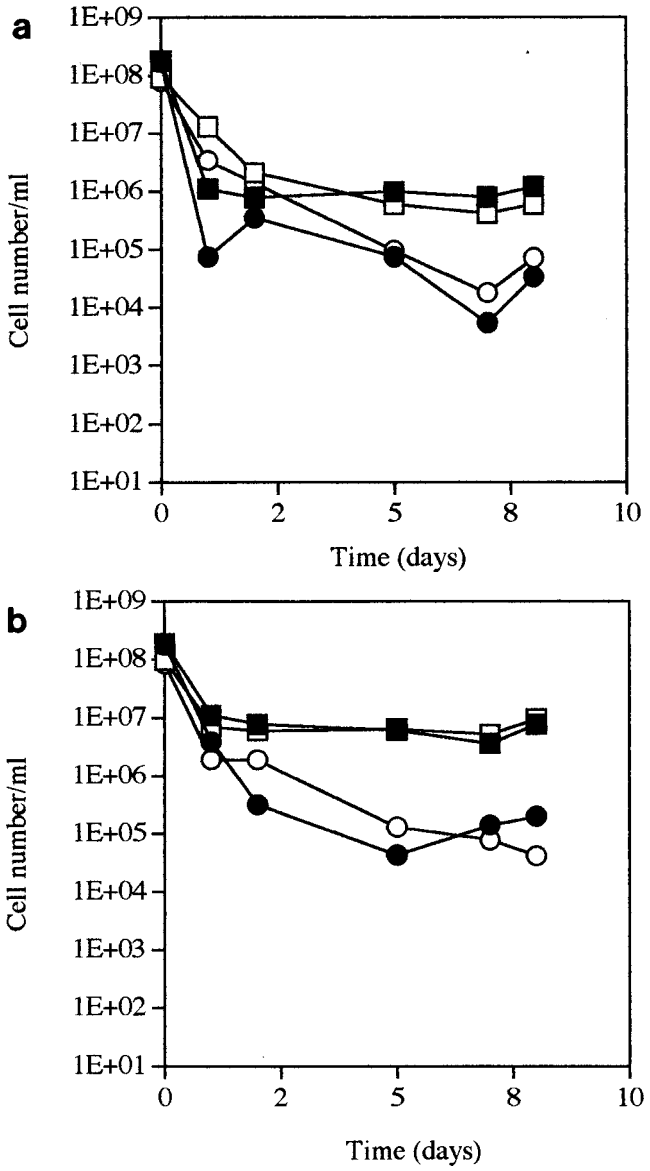

FIG. 4. Entrance of logarithmic- and stationary-phase cells of both the opaque and translucent morphotypes of *V. vulnificus* into a VBNC state in 10 to 18°C estuarine waters. Shown are results of total direct count (squares) and DVC (circles) assays of both the opaque (closed symbols) and translucent (open symbols) morphotypes of each strain. Panels: a, logarithmic-phase cells; b, stationary-phase cells.

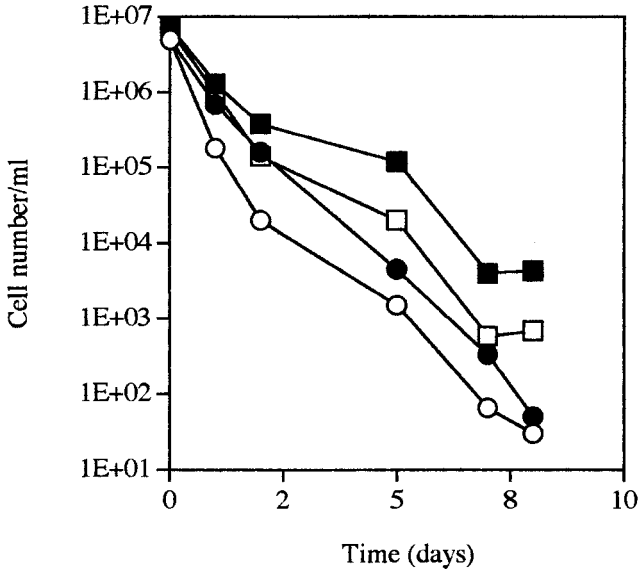

FIG. 5. Entrance of logarithmic- and stationary-phase cells of both the opaque and translucent morphotypes of *V. vulnificus* into a VBNC state in laboratory ASW microcosms. Shown are results of plate count assays of stationary-phase (squares) and logarithmic-phase (circles) cells of both the opaque (closed symbols) and translucent (open symbols) morphotypes.

to room temperature (ca. 22°C) in the laboratory. While DVC data indicated continued viability, no resuscitation was observed, suggesting the possibility that natural water bodies, such as that employed in our studies, contain some factor(s) which facilitates resuscitation.

This resuscitation study was repeated in October with cells that had been in a VBNC state for 7 days and with less than 0.005 CFU/ml (when 200 ml was filtered). As shown in Fig. 10, rapid resuscitation again occurred in situ, reaching cell levels of $10^2 \times 10^4$ to 4.7×10^4 CFU/ml by the first (12-h) sampling and levels equal to the original DVCs (3.1×10^5 cells/ml for opaque cells and 2.0×10^5 cells/ml for translucent cells) by 48 h. This increase in population density is believed to be due to true resuscitation, and not cell growth, as fully culturable cells diluted to ca. 10 CFU/ml and placed in the same environment required 60 h to achieve populations of only ca. 4×10^5 CFU/ml (Fig. 11). These late-log-phase cells, when placed into estuarine water, also demonstrated a 12- to 24-hour lag period prior to growth (Fig. 11). In contrast, VBNC cells, which were in a state of nongrowth due to 19 days of refrigeration, did not appear to undergo such a lag but instead appeared to rapidly undergo conversion to the culturable state.

In contrast to the previous study, population growth was

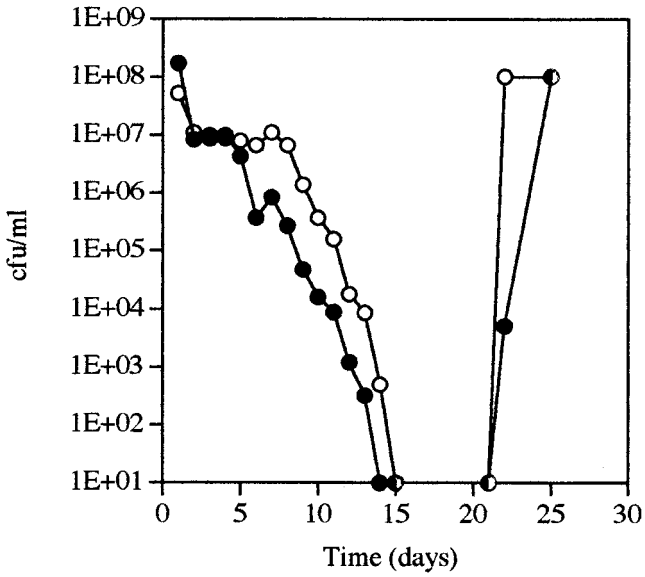

FIG. 6. Resuscitation of *V. vulnificus* cells from a VBNC state in estuarine waters during September and October. Shown are plate counts for the opaque (●) and translucent (○) morphotypes. Cells were induced to enter a VBNC state through incubation at 5°C in laboratory microcosms and then placed in estuarine waters with temperatures of 22 to 24°C.

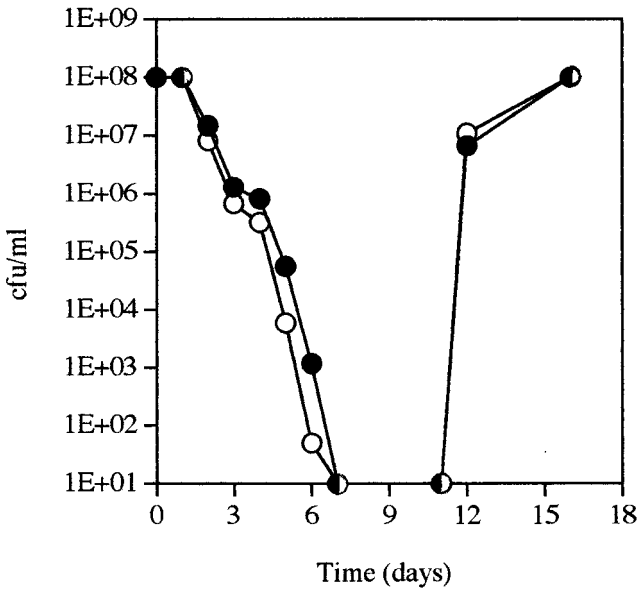

FIG. 7. Resuscitation of *V. vulnificus* cells from a VBNC state in estuarine waters during November. Shown are plate counts of the opaque (●) and translucent (○) morphotypes. Cells were induced to enter a VBNC state through incubation at 5°C in laboratory microcosms and then placed in estuarine waters with temperatures of 16 to 19°C.

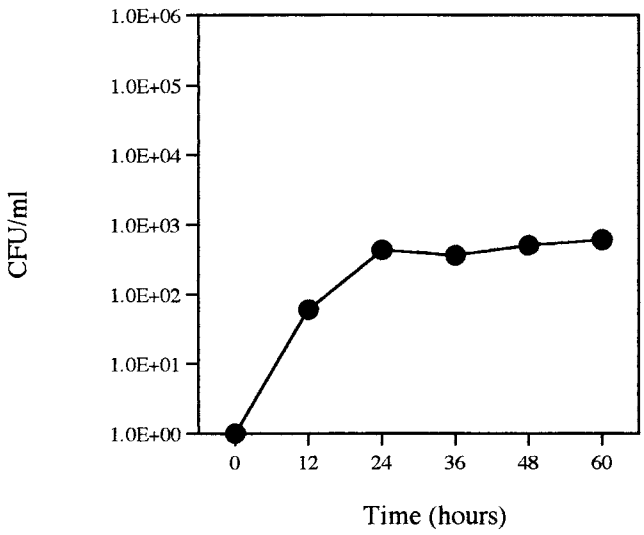

FIG. 9. Incubation of fully culturable cells (diluted to a population density of ca. 1 CFU/ml) of *V. vulnificus* (opaque morphotype) in estuarine waters. This study was performed concurrently with that whose results are shown in Fig. 8.

seen to occur in these chambers containing VBNC cells, presumably after resuscitation occurred, with final culturable cell numbers exceeding the total original AODC levels of 3.5×10^5 to 7.7×10^5 cells/ml by 1 to 1.5 logs by ca. 48 h of in situ incubation (Fig. 10). As the nutrient contamination resulting from preparation of the inoculum would have been less than 0.15 mg/ml, such growth is most likely due to utilization of the nutrients naturally present in these estuarine waters.

In this study, incubation of the VBNC cell populations in the laboratory at room temperature did result in resuscitation, although the final levels were ca. 1 log lower (3.2×10^5 and 2×10^6 CFU/ml for opaque and translucent strains, respectively) than those observed for the same cells incubated in situ (4.6×10^6 and 6.2×10^6 CFU/ml for the opaque and translucent strains, respectively). The difference is likely due to utilization of estuarine nutrients not available to laboratory-incubated cells.

Both opaque and translucent culturable cells, which had also been placed as controls into separate environmental chambers at the estuarine site, remained fully culturable at all sample times (data not shown).

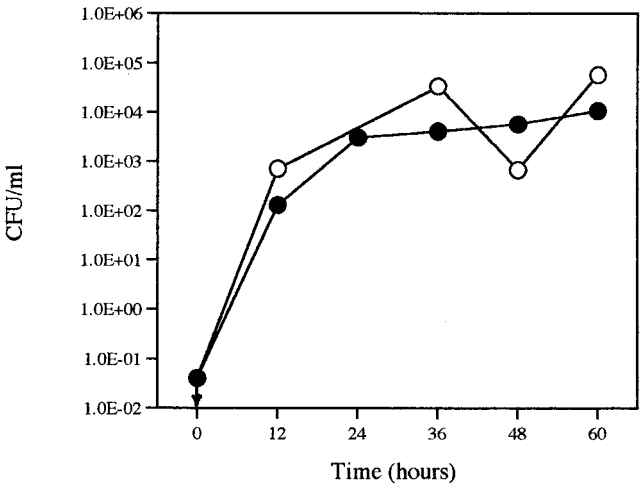

FIG. 8. Resuscitation of *V. vulnificus* cells from a VBNC state ($<4 \times 10^{-2}$ culturable cells were present) in estuarine waters during August. Shown are plate counts of the opaque (●) and translucent (○) morphotypes and both the total counts and DVCs of the nonculturable populations of each morphotype. A mean water temperature of 25°C existed during this study.

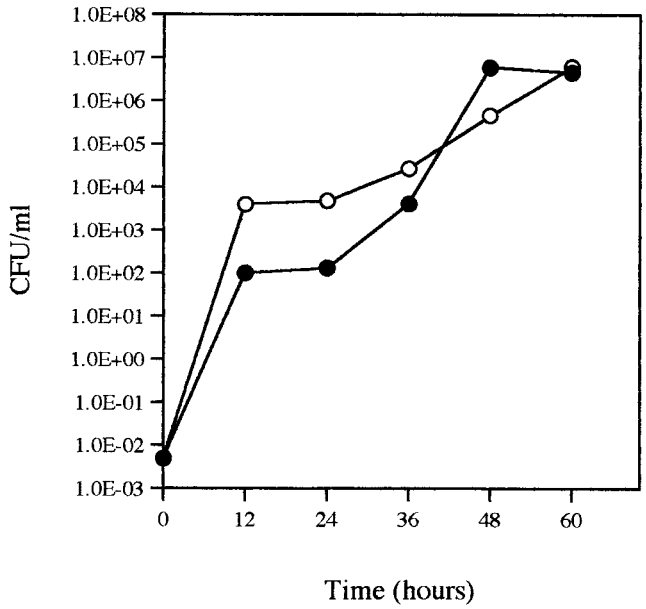

FIG. 10. Resuscitation of *V. vulnificus* cells from a VBNC state ($<5 \times 10^{-3}$ culturable cells were present) in estuarine waters during October. Shown are plate counts of the opaque (●) and translucent (○) morphotypes. A mean water temperature of 23°C existed during this study.

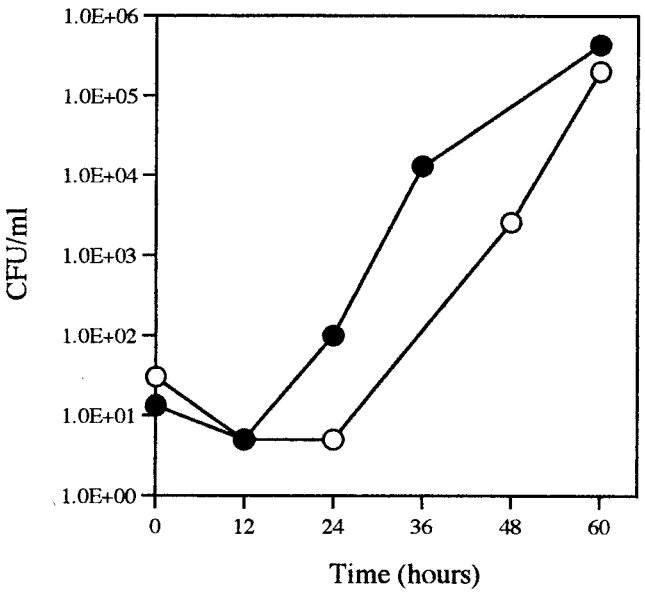

FIG. 11. Incubation of fully culturable cells (diluted to a population density of ca. 10 CFU/ml) of the opaque (●) and translucent (○) morphotypes of *V. vulnificus* in estuarine waters. This study was performed concurrently with that whose results are shown in Fig. 10.

DISCUSSION

The use of membrane diffusion chambers allowed us to monitor the fate of *V. vulnificus* cells in an estuarine environment. Further, the use of *V. vulnificus* CVD713 greatly simplified monitoring of the culturability of this bacterium, as the colonies this strain forms are bright blue on Tn agar because of Tn*phoA*-mediated hydrolysis of the alkaline phosphatase substrate present in the medium. As a consequence, we could be confident that the cells being cultured were the same as those introduced into the chamber, an especially important consideration during resuscitation studies.

The plate counts obtained when culturable cells were placed into cold estuarine waters indicated that cells of *V. vulnificus* were able to rapidly enter into a VBNC state in this estuarine environment. The time course for these cells to become nonculturable was not greatly different from that observed when *V. vulnificus* cells are incubated in ASW in the laboratory at 5°C. However, our previous laboratory studies have indicated that the growth phase of the cells prior to temperature downshift greatly affected the time required to become nonculturable. Oliver et al. (16) reported that cells taken from the stationary phase required twice as long to become nonculturable as did logarithmic-phase cells, a result believed to be related to the production of stationary-phase-induced stress proteins. A similar finding was reported by Weichart et al. (23). In contrast, no difference was observed between logarithmic- and stationary-phase cells of *V. vulnificus* regarding their rate of nonculturability when incubated in estuarine waters (Fig. 3). The reason for the difference between our laboratory and in situ studies is not known.

Another important difference between our previous laboratory studies and the estuarine studies presented here is the temperature which was found to induce a VBNC state in *V. vulnificus*. Our laboratory studies had previously indicated that temperatures of 5 to 10°C induces a VBNC state in this bacterium when it is incubated in ASW (24) or elevated-nutrient solutions (17). In contrast, the studies reported here indicate that *V. vulnificus* enters a VBNC state in estuarine waters which, at least at times, had reached a temperature of 18°C. While the average water temperatures of the study site were 10 to 15°C for the four separate studies reported here on cells entering the VBNC state, it appears that cells of *V. vulnificus* may be induced to enter this state in the natural environment at a temperature higher than that of laboratory microcosms. The level or form of nutrients present in these estuarine environments may also have an effect on induction temperatures. All temperatures which induce the VBNC state, however, appear to result in the same response, as both our laboratory (Fig. 5) and in situ (Fig. 1 to 3) cultures became nonculturable when incubated at any temperature between 5 and 18°C. The reasons for the difference between our laboratory and estuarine studies are not known but likely depend on the constantly changing biological, physical, and chemical factors present in estuarine waters. It is interesting, however, that Tilton and Ryan (22) reported their inability to isolate *V. vulnificus* from Long Island Sound waters when water temperatures were below ca. 17°C, a temperature very similar to that reported here for our estuarine studies. Similar results have been reported by O'Neill et al. (18) in New England waters and Kaysner et al. (3) for west coast waters.

Virulent cells of *V. vulnificus* are known to possess an antiphagocytic capsule which results in opaque colonies on routine laboratory media. Spontaneous mutants arise, however, which lack this capsule and which, as a consequence, produce translucent colonies and are avirulent (21). As we have seen in our laboratory studies (24), possession of this capsular material did not appear to affect either the time required to enter the VBNC state in estuarine waters nor the ability of the cells to resuscitate. It appears from both electron microscopic studies (6) and capsule-specific antibody studies (15a) that the capsule is not lost during entry of *V. vulnificus* into the VBNC state in the laboratory and that cells in the VBNC state maintain virulence for laboratory animals (15). Thus, the finding in the present study that presence or absence of this capsule does not determine which morphotype survives water temperature fluctuations in the natural environment is significant.

Even for those studies reported here which did not result in complete nonculturability of the cells (Fig. 3), it is clear from the total and DVC data (Fig. 1, 2, and 4) that a majority of the cells had entered a VBNC state. Considering our four separate studies on entry into this state, including both logarithmic- and stationary-phase cells and both encapsulated and nonencapsulated cells of each growth phase, our data clearly indicate the ability of *V. vulnificus* to enter a VBNC state in this estuarine environment.

Studies on the ability of *V. vulnificus* cells to exit the VBNC state when incubated in warm estuarine waters employed cells which had been refrigerated for an additional 4 to 6 days after they had entered this state. Despite this treatment, the VBNC cells were seen to rapidly develop into large populations of culturable cells (ca. 10^7 to 10^8 CFU/ml), producing bright blue colonies on Tn agar, typically within the first 24 h of in situ incubation (Fig. 6–8 and 10).

Some studies, even with *V. vulnificus*, have argued that resumption of culturability following a temperature upshift can be accounted for by "regrowth" of a few residual culturable cells remaining among the nonculturable population (19a, 23). However, these studies have all been conducted with laboratory microcosms. Our belief that this rapid development of culturability represents true resuscitation of the VBNC population to the actively metabolizing, culturable state and is not a result of growth of a few residual cells present in the membrane diffusion chambers is based on the following observa-

tions. In our summer studies (Fig. 8 and 10), we employed a *V. vulnificus* population that contained $<4 \times 10^{-2}$ CFU/ml. Thus, there should not have been any culturable cells in the entire diffusion chamber (<1 CFU in the 25-ml chamber volume). Even if a culturable cell had been present in a chamber, it would have had to demonstrate an extremely rapid rate of growth in these estuarine waters to reach the large culturable populations which resulted by the first samplings. Studies on control populations composed of culturable cells diluted to between 1 and 10 CFU/ml (Fig. 9 and 11) suggest that reaching these populations in such a short time period is not likely.

That the DVC of a VBNC cell population is the most accurate estimation of cell viability is suggested by our August resuscitation study (Fig. 8), which indicated that VBNC cells could resuscitate only to that level. However, our data suggest that once a VBNC population does resuscitate, sufficient nutrient may be present in the estuarine waters to support a cell population greater than that originally present in the chambers. Cells in our October study ultimately exceeded even the total cell count by approximately 1 log. The ability of cells to grow in dilute nutrients is well established, and Camper et al. (1) have demonstrated growth of several coliforms at <1 mg of dissolved organic carbon per liter. Laboratory resuscitation of *V. vulnificus* cells from a VBNC state by a temperature upshift has been reported (12), and we have commonly observed this event in our laboratory. In the present study, we included laboratory resuscitation studies as a comparison to the in situ resuscitation experiments. While rapid resuscitation was observed in one of our control studies, the final population density reached was less than that observed in the in situ studies. While this may be due simply to the lack of exogenous nutrients in the laboratory microcosm, it is interesting that resuscitation was not observed in our first study, suggesting the possibility that natural water bodies, such as that employed in our studies, contain some factor(s) which facilitates resuscitation. Alternatively, it is possible that some inherent difference between the closed centrifuge tubes used in the first resuscitation study and the membrane diffusion chambers employed in the second might account for our results.

This is the first study which has monitored both entry into and exit from a VBNC state by a bacterium in a natural environment. Our results suggest that entrance into this state is likely a natural response to adverse environmental factors (in this case, low temperature), and thus the VBNC state likely represents another survival strategy for aquatic bacteria. Cells of *V. vulnificus* in this state appear able to rapidly resuscitate to the actively metabolizing, culturable state at temperatures of 16°C and above. Such a response is likely essential to the ability of the population to react rapidly to removal of the stress, thus allowing the cells' continued participation in the overall ecology of the environment. We have initiated a program of study to investigate both starvation-induced (10) and cold shock-induced (8) stress proteins in *V. vulnificus* and the role such stresses play in the VBNC response.

ACKNOWLEDGMENTS

This study was supported by grants from the National Institutes of Health (AI31216) and the U.S. Department of Agriculture (91-37201-6877).

We thank Jim Merrit for providing research space at the Marine Research Laboratory, University of North Carolina at Wilmington.

REFERENCES

1. **Camper, A. K., G. A. McFeters, W. G. Characklis, and W. L. Jones.** 1991. Growth kinetics of coliform bacteria under conditions relevant to drinking water distribution systems. Appl. Environ. Microbiol. **57:**2233–2239.
1a. **DePaola, A., G. M. Capers, and D. Alexander.** 1994. Densities of *Vibrio vulnificus* in the intestines of fish from the U.S. Gulf Coast. Appl. Environ. Microbiol. **60:**984–988.
2. **Groubert, T. N., and J. D. Oliver.** 1994. Interaction of *Vibrio vulnificus* and the eastern oyster, *Crassostrea virginica*. J. Food Prot. **57:**224–228.
3. **Kaysner, C. A., C. A. Abeyta, M. M. Wekell, A. DePaola, Jr., R. F. Stott, and J. M. Leitch.** 1987. Virulent strains of *Vibrio vulnificus* isolated from estuaries of the United States west coast. Appl. Environ. Microbiol. **53:**1349–1351.
4. **Kjelleberg, S., M. Hermansson, and P. Mårdén.** 1987. The transient phase between growth and nongrowth of heterotrophic bacteria, with emphasis on the marine environment. Annu. Rev. Microbiol. **41:**25–49.
5. **Kogure, K., U. Simidu, and N. Taga.** 1979. A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. **25:**415–420.
6. **Linder, K., and J. D. Oliver.** 1989. Membrane fatty acid and virulence changes in the viable but nonculturable state of *Vibrio vulnificus*. Appl. Environ. Microbiol. **55:**2837–2842.
7. **McFeters, G. A., and D. G. Stuart.** 1972. Survival of coliform bacteria in natural waters: field and laboratory studies with membrane filter chambers. Appl. Environ. Microbiol. **24:**805–811.
8. **McGovern, V. P., and J. D. Oliver.** 1995. Induction of cold-responsive proteins in *Vibrio vulnificus*. J. Bacteriol. **177:**4131–4133.
9. **Morita, R. Y.** 1993. Bioavailability of energy and the starvation state, p. 1–24. *In* S. Kjelleberg (ed.), Starvation in bacteria. Plenum Press, New York.
10. **Morton, D., and J. D. Oliver.** 1994. Induction of carbon starvation proteins in *Vibrio vulnificus*. Appl. Environ. Microbiol. **60:**3653–3659.
11. **Murphy, S. E., and J. D. Oliver.** 1992. Effects of temperature abuse on *Vibrio vulnificus* in oysters. Appl. Environ. Microbiol. **58:**2771–2775.
12. **Nilsson, L., J. D. Oliver, and S. Kjelleberg.** 1991. Resuscitation of *Vibrio vulnificus* from the viable but nonculturable state. J. Bacteriol. **173:**5054–5059.
13. **Oliver, J. D.** 1989. *Vibrio vulnificus*, p. 569–600. *In* M. P. Doyle (ed.), Foodborne bacterial pathogens. Marcel Dekker, Inc., New York.
14. **Oliver, J. D.** 1993. Formation of viable but nonculturable cells, p. 239–272. *In* S. Kjelleberg (ed.), Starvation in bacteria. Plenum Press, New York.
15. **Oliver, J. D., and R. Bockian.** 1995. In vivo resuscitation, and virulence towards mice, of viable but nonculturable cells of *Vibrio vulnificus*. Appl. Environ. Microbiol. **61:**2620–2623.
15a. **Oliver, J. D., and R. A. Duerbeck.** Unpublished data.
16. **Oliver, J. D., L. Nilsson, and S. Kjelleberg.** 1991. The formation of nonculturable cells of *Vibrio vulnificus* and its relationship to the starvation state. Appl. Environ. Microbiol. **57:**2640–2644.
17. **Oliver, J. D., and D. Wanucha.** 1989. Survival of *Vibrio vulnificus* at reduced temperatures and elevated nutrient. J. Food Safety **10:**79–86.
18. **O'Neill, K. R., S. H. Jones, and D. J. Grimes.** 1992. Seasonal incidence of *Vibrio vulnificus* in the Great Bay estuary of New Hampshire and Maine. Appl. Environ. Microbiol. **58:**3257–3262.
19. **Östling, J., L. Holmquist, K. Flärdh, B. Svenblad, Å. Jouper-Jaan, and S. Kjelleberg.** 1993. Starvation and recovery of *Vibrio*, p. 103–128. *In* S. Kjelleberg (ed.), Starvation in bacteria. Plenum Press, New York.
19a. **Ravel, J., R. T. Hill, I. T. Knight, C. R. Dubois, and R. R. Colwell.** 1993. Recovery of *Vibrio cholerae* from the viable but nonculturable state, abstr. Q6, p. 347. Abstracts of the 93rd General Meeting of the American Society for Microbiology 1993. American Society for Microbiology, Washington, D.C.
20. **Roper, M. M., and K. C. Marshall.** 1979. Effects of salinity on sedimentation of particulates on survival of bacteria in estuarine habitats. Geomicrobiol. J. **1:**103–116.
21. **Simpson, L. M., V. K. White, S. F. Zane, and J. D. Oliver.** 1987. Correlation between virulence and colony morphology in *Vibrio vulnificus*. Infect. Immun. **55:**269–272.
22. **Tilton, R. C., and R. W. Ryan.** 1987. Clinical and ecological characteristics of Vibrio vulnificus in the northeastern United States. Diagn. Microbiol. Infect. Dis. **6:**109–117.
23. **Weichart, D., J. D. Oliver, and S. Kjelleberg.** 1992. Low temperature induced nonculturability and killing of *Vibrio vulnificus*. FEMS Microbiol. Lett. **100:**205–210.
24. **Wolf, P., and J. D. Oliver.** 1992. Temperature effects on the viable but nonculturable state of *Vibrio vulnificus*. FEMS Microbiol. Ecol. **101:**33–39.
25. **Wright, A. C., L. M. Simpson, J. D. Oliver, and J. G. Morris, Jr.** 1990. Phenotypic evaluation of acapsular transposon mutants of *Vibrio vulnificus*. Infect. Immun. **58:**1769–1773.