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# Resuscitation of *Vibrio vulnificus* from the Viable but Nonculturable State

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**Stationary-phase-grown cells of the estuarine bacterium *Vibrio vulnificus* became nonculturable in nutrient-limited artificial seawater microcosms after 27 days at 5°C. When the nonculturable cells were subjected to temperature upshift by being placed at room temperature, the original bacterial numbers were detectable by plate counts after 3 days, with a corresponding increase in the direct viable counts from 3% to over 80% of the total cell count. No increase in the total cell count was observed during resuscitation, indicating that the plate count increases were not due to growth of a few culturable cells. Chloramphenicol and ampicillin totally inhibited resuscitation of the nonculturable cells when added to samples that had been at room temperature for up to 24 h. After 72 h of resuscitation, the inhibitors had an easily detectable but reduced effect on the resuscitated cells, indicating that protein and peptidoglycan synthesis were still ongoing. Major changes in the morphology of the cells were discovered. Nonculturable cells of *V. vulnificus* were small cocci (approximately 1.0 μm in diameter). Upon resuscitation, the cells became large rods with a size of mid-log-phase cells (3.0 μm in length). Four days after the cells had become fully resuscitated, the cell size had decreased to approximately 1.5 μm in length and 0.7 μm in width. The cells were able to go through at least two cycles of nonculturability and subsequent resuscitation without changes in the total cell count. This is the first report of resuscitation, without the addition of nutrient, of nonculturable cells, and it is suggested that temperature may be the determining factor in the resuscitation from this survival, or adaptation, state of certain species in estuarine environments.**

*Vibrio vulnificus* is an estuarine bacterium occurring in coastal waters in many parts of the world (17). During warm water seasons, the bacterium is readily detectable by plating samples onto solid nutrient media. However, the organism can usually not be isolated from water samples that are collected during the winter (17), a situation similar to that reported for *V. parahaemolyticus* (4), *V. cholerae* (5), and *V. mimicus* (3). It has recently been demonstrated that this inability to culture certain *Vibrio* species from low-temperature environments is due not to cell death but to the viable but nonculturable state, which we define as an inability of cells to produce colonies on appropriate solid media even following prolonged incubation (5, 8, 22, 26). With respect to *V. vulnificus*, the cells easily become nonculturable when incubated at a water temperature of 5°C (25). The bacterium enters into such a state whether it is present in nutrient-rich media or in nutrient-free saltwater microcosms (8, 19), indicating that the downshift in temperature plays the major role in inducing the nonculturable state in this particular *Vibrio* species.

Various other gram-negative bacteria are also known to enter into a state of nonculturability, often induced when the bacteria are exposed to adverse environmental conditions. This has been demonstrated for several human pathogens, such as *Escherichia coli*, *V. cholerae*, and *Salmonella enteritidis* (22), *Shigella sonnei* and *S. flexneri* (5), and *Campylobacter jejuni* (21). When the cells have entered the state of nonculturability, neither plating onto solid media nor inoculation into liquid media reveals the presence of viable bacteria. Thus, standard bacteriological culture methods are inadequate to detect such cells, and special resuscitation methods are required.

Whereas successful in vitro resuscitation and growth of nonculturable cells of *S. enteritidis* have been reported by adding nutrient media of different strengths and incubating the sample for 25 h (22), it would be preferable to determine the accurate number of bacteria in a sample without nutrient addition. Any such manipulation could considerably alter the composition of the flora present or the bacterial numbers. In the studies presented here, nonculturable cells of *V. vulnificus* were used in an attempt to identify a method for resuscitating and recovering organisms in this state, without the addition of nutrients. The studies were based on the assumptions that the bacterium responds to a temperature downshift by entering a viable but nonculturable state and that an upshift in temperature would release the organism from low-temperature stress, thereby promoting resuscitation to the original cell state and number. As our studies suggested that resuscitation from the nonculturable state is an active process, we also examined some metabolic changes occurring during resuscitation by the use of inhibitors of protein and peptidoglycan synthesis.

## MATERIALS AND METHODS

**Organism and microcosm conditions.** A nonencapsulated strain of *V. vulnificus* C7184t (23) was used in these studies. Stationary-phase cells were obtained by growing the organism in VNSS broth (9) without soluble starch for 24 h at room temperature with shaking. The culture was diluted 100-fold by adding 5 ml to 495 ml of room temperature sterile nine-salt solution (NSS, pH 7.8; 9) in acid-washed 1-liter screw-cap flasks. The initial cell number in the microcosms was approximately $2.0 \times 10^6$ CFU/ml. The flasks were

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chilled to 5°C and maintained at that temperature throughout the experiment as described by Linder and Oliver (8).

The influence of the cells' physiological state on resuscitation was studied. Mid-phase cells, cells in early stationary phase, and cells in late stationary phase (a 24-h culture), grown in VNSS broth without starch, were diluted 100-fold in artificial seawater (ASW) microcosms and placed at 5°C. Plate counts were determined during the low-temperature (5°C) incubation as well as during resuscitation of the nonculturable cells, as described above. To determine any possible effects of the salt composition of the microcosm menstruum, cells were incubated in either NSS or ASW and then treated as described above. Resuscitation of the nonculturable cells were performed by placing samples at room temperature. The recovery of the cells was monitored as described above.

**Cell enumeration and viability measurements.** At the onset of suspending the cells in NSS, $T_0$ (inoculation time), the samples were diluted in room temperature NSS. When at 5°C, the samples from the flask were aseptically removed and diluted in cold NSS (5°C) to avoid heat shocking the cells. From appropriate dilutions, 10-μl aliquots were plated onto cold (5°C) LB20 agar plates by the drop plate method (6). These nutrient agar plates contained 20 g of NaCl, 5 g of yeast extract, 10 g of tryptone, 15 g of agar, 10 μg of $FeSO_4 \cdot 7H_2O$, 0.2033 g of $MgCl_2 \cdot 6H_2O$, and 0.0485 g of $CaCl_2 \cdot 2H_2O$ per 1,000 ml of distilled water. The salts were added as filter-sterilized solutions. The plates were incubated at room temperature for 72 h before the number of colonies present was counted. Although this incubation period was routinely used, prolonged incubation (up to 12 days) failed to result in colony development.

To determine whether culturable cells persisted by the end of the incubation period, 10 ml of the microcosm was filtered through a 0.22-μm-pore-size membrane filter (Millipore Corp.), which was placed on the solid medium and observed for growth. When less than 0.1 cell per ml of microcosm was culturable, the cells were considered to be in the nonculturable state.

Total bacterial counts were performed by staining formalin-fixed cells with a 0.1% acridine orange solution as described by Oliver (16). Direct viable acridine orange direct counts (AODCs) were determined by the *p*-iodonitrotetrazolium violet (INT) assay (27) as modified by Oliver and Wanucha (19) except that a 2- to 3-h incubation time was used. INT (Sigma Chemical Co.) is an electron acceptor which diverts electrons from an active electron transport chain. Reduction of the soluble INT by metabolizing cells leads to the formation of insoluble INT-formazan, resulting in a visible precipitate in the cell membrane.

**Resuscitation conditions.** When less than 0.1 cell per ml was culturable, 10-ml samples were removed aseptically from the microcosm and transferred to sterile 15-ml test tubes. The tubes were placed at room temperature in a static state. Samples were processed for enumerating plate counts, total cell counts, and INT counts every 24 h as described above.

**Microscopic observations.** During incubation of the microcosms at 5°C as well as during the subsequent resuscitation at room temperature, the cells were observed microscopically. Changes in morphology and size were monitored.

**Effects of chloramphenicol and ampicillin on resuscitation.** Ten-milliliter samples were removed from the microcosms, transferred to 15-ml sterile test tubes, and placed at room temperature as described above. Chloramphenicol or ampicillin, at a final concentration of 100 μg/ml, was added to samples that had been resuscitated at room temperature for 0, 8, 24, 48, or 72 h prior to addition of the inhibitors. The effects of the inhibitors on the cells were monitored by plate counts and total counts.

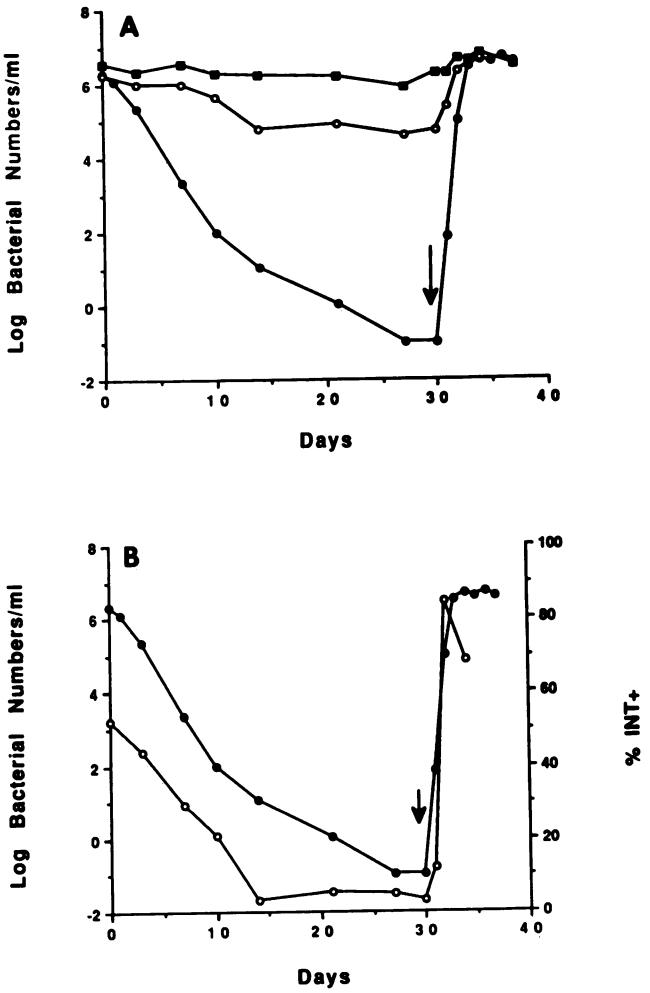


FIG. 1. Effects of temperature down- and upshifts on stationary-phase cells of *V. vulnificus* in NSS microcosms. (A) Plate counts (●), AODCs (■), and total viable counts determined by the INT assay (○); (B) plate counts (●) and percent viable cells (○) determined by the INT assay (in relation to the AODCs). The arrow indicates when the 5°C microcosms were shifted to room temperature.

## RESULTS

**Resuscitation of viable but nonculturable cells: activity and morphological changes.** Plate counts indicated that low-temperature incubation at 5°C resulted in nonculturability in approximately 27 days (Fig. 1). Prolonged incubation (up to 12 days) of these cells on LB20 agar plates did not result in the appearance of colonies. Despite this decline, a significant population of active cells remained, as determined by the INT assay. When there was less than 0.1 plateable cell per ml in the microcosm, 3% of the cells, i.e., $6 \times 10^4$ cells per ml, were still active (Fig. 1). During this time there was no decline in the total number of cells present in the microcosms. When the cells were shifted to room temperature (23°C), some cells were plateable after having been held for

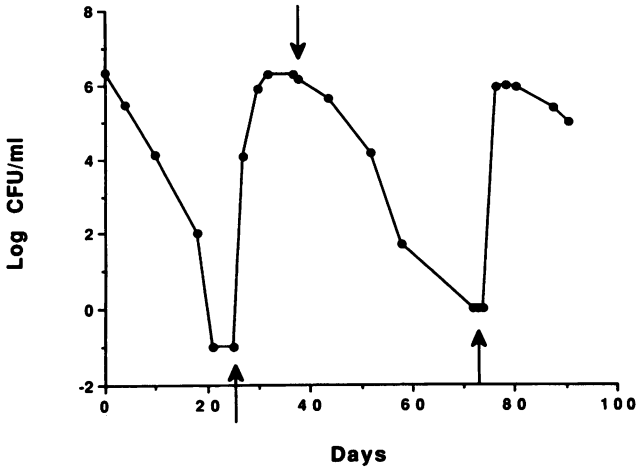


FIG. 2. Plate counts for cells entering the nonculturable state during temperature downshift and subsequent resuscitation following temperature upshift. The results are representative for log-phase and stationary-phase cells in ASW and for stationary-phase cells in NSS. The arrows indicate temperature down- and upshifts.

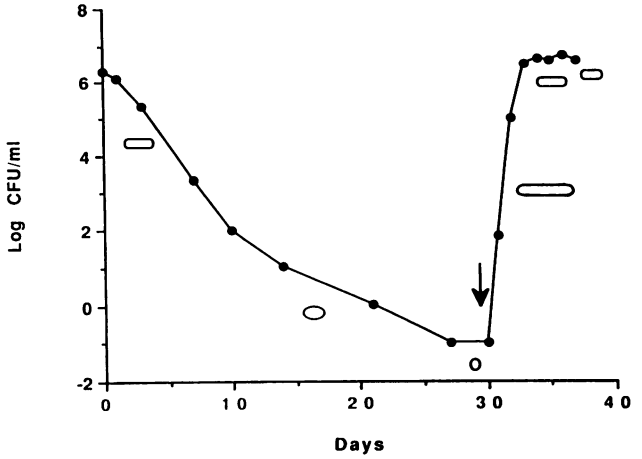


FIG. 3. Changes in cell morphology during temperature downshift and subsequent resuscitation of the nonculturable cells. ●, plate counts.

2 days at the higher temperature, and numbers of plateable cells reached the same level as in the original cultures after 3 days (Fig. 1A). There was a corresponding large increase in drect viable cell counts (INT-positive cells) over a period of 48 h from the time at which the samples were shifted to the higher temperature (Fig. 1B). Presumably as a result of starvation-induced reductive division, a slight increase in total cell counts was noted after 72 h. There were no major differences in the rate of resuscitation between cells in NSS or ASW or between cells in log or stationary phase when inoculated into the microcosms.

In an attempt to determine whether cells were capable of responding to more than one cycle of temperature up- and downshift, cells were exposed alternatively to 5°C and room temperature. Figure 2 shows that cells which had been nonculturable for at least 4 days responded rapidly to the initial temperature upshift, with complete plateability observed by 7 days. After an additional 5 days, these resuscitated cells were returned to 5°C. A second round of nonculturability was seen which developed at a rate approximately the same as that seen for the first downshift (Fig. 2). These cells could again be resuscitated by incubation at room temperature. We did not attempt to establish additional cell cycles in these studies.

Morphological changes paralleled changes in culturability and resuscitation (Fig. 3). The cells inoculated into the microcosms were rods with a size of approximately 1.5 by 0.7 μm. Nonculturable cells of *V. vulnificus* were small cocci with a diameter of 0.8 to 1.0 μm. Upon 48 h of resuscitation, the cocci developed into rods approximately the size of mid-log cells in a nutrient medium, averaging 3.0 by 0.7 μm. After 4 days at room temperature, the rods were the size of stationary-phase cells; after an additional 3 days at room temperature, the cells were small coccobacilli with a size of 1.0 to 1.5 by 0.6 to 0.7 μm. The cell volume increased from approximately 0.3 $\mu m^3$ for the cocci to approximately 1.2 $\mu m^3$ for the rods appearing after 48 h at room temperature. Prolonged incubation at 5°C or after resuscitation at room temperature for longer periods of time will result in cell sizes smaller than those presented here (12).

**Effects of chloramphenicol and ampicillin exposure on resuscitating cells.** In a separate study, the effects on resuscitation by the protein synthesis inhibitor chloramphenicol and the peptidoglycan synthesis inhibitor ampicillin were investigated. As previously observed, the control cells (no inhibitors added) fully resuscitated to the original number of cells after 72 h at room temperature, with cells initially becoming plateable after incubation for 24 h at the elevated temperature (Fig. 4A). When the two inhibitors were added immediately at temperature upshift or when the cells had been at room temperature for 8 h, no resuscitation of the cells was achieved. When the inhibitors were added to samples that had been at room temperature for 24 h, resuscitation of the cells was clearly discontinued (Fig. 4B). Whereas the cells had initiated resuscitation and a small fraction were plateable on solid media, <10 cells per ml were plateable after the addition of the inhibitors. The effects of the inhibitors when added to cells that had been at room temperature for 48 h are presented in Fig. 4C. By 48 h, the cells had almost fully resuscitated at the time of antibiotic addition. Again, the inhibitors had an effect on resuscitation but not to the same extent as seen with the 24-h samples. After 72 h at room temperature, addition of the inhibitors resulted in only a slight inhibition of the resuscitation of cells, suggesting that a small portion of the population was still dependent on protein and peptidoglycan synthesis (Fig. 4D). The total cell counts remained constant throughout all of these experiments and were not affected by addition of the inhibitors, indicating that cell lysis was not induced.

## DISCUSSION

*V. vulnificus* enters a viable but nonculturable state when incubated at 5°C in nutrient-limited ASW microcosms. In this study, it took approximately 27 days for the stationary-phase grown cells to become nonculturable, a time consistent with the literature (8).

On resuscitation, an increase in plate counts from less than 0.1 cell per ml to the original cell density was observed to occur. Until the point of complete resuscitation, no increase in total cell counts was observed. It should be noted, however, that if any cells in our microcosms remained culturable, an increase in plate counts from values below our level of detection to $2 \times 10^6$ would be reflected in

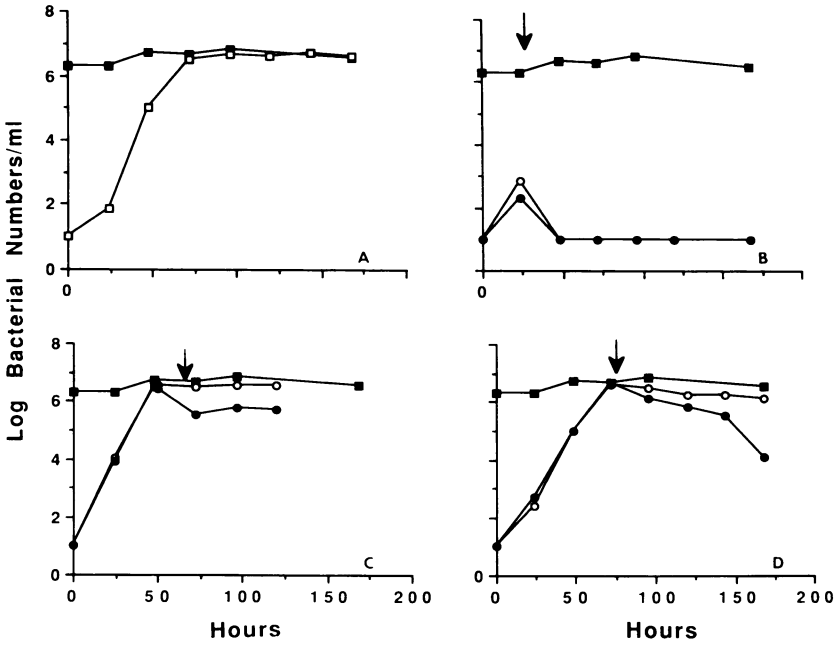


FIG. 4. Effects of chloramphenicol and ampicillin on resuscitation of nonculturable cells. (A) Plate counts (□) and total counts (■) for control cells (no antibiotic additions); (B to D) total counts (■) and plate counts for samples to which ampicillin (○) or chloramphenicol (●) was added. The arrows indicate the time of addition of the inhibitors.

the total cell count increasing from the initial $2 \times 10^6$ to only $4 \times 10^6$, an increase likely not to be readily apparent. Evidence that the plate count increase was not due to such growth of any residual, culturable cells was provided by our microscopic observations.

Stationary-phase cells of *V. vulnificus* are small rods (1.0 to 1.5 μm in length and 0.5 to 0.7 μm in width). When incubated at a low temperature in a starvation medium, these cells gradually form small cocci (see also reference 8) with a diameter usually not exceeding 0.8 μm. Upon resuscitation, as seen in this study, the cocci increased in size to form rods averaging 3 μm in length and 0.7 μm in width. This occurred after 48 h of incubation at room temperature. The cell volume increased four times during the first 48 h of resuscitation, possibly as a result of uptake of water by the cells. The possibility that cells are in a dehydrated state, as evidenced by a highly condensed nucleoid (11), under starved or nonculturable condition needs to be addressed in future studies. Since no cocci were present in the samples after 48 h, it is concluded that the primarily large rods comprising the resuscitated samples originated from the nonculturable coccus-shaped cells, supporting the idea of resuscitation as opposed to growth of a few residual culturable cells. After 96 h at room temperature, small rods (1.2 to 1.8 μm in length and 0.6 to 0.7 μm in width) predominated, with some small cocci also visible. This observation suggests that the large cells seen at 48 h decreased in size as a result of reductive division, concomitant with biomass decrease (2, 10, 13, 15), in response to room temperature starvation conditions.

It should be noted that the INT method for determining, through direct examination, the number of respiring cells in a population may under certain conditions significantly underestimate these numbers. As is evident from our study, whereas only 3% of the cells exhibited detectable INT-formazan deposits (Fig. 1B), 100% of these same cells were plateable following room temperature resuscitation. Even though in our study an extended (2- to 3-h) incubation period was used, and although this assay remains one of the few capable of detecting viable but nonculturable cells, caution must be used in interpretation of results based on this assay.

We included two cycles of nonculturability and subsequent resuscitation in this study, with the cells demonstrating the same morphological changes as described above during the second round of resuscitation. That two cycles of nonculturability and resuscitation occurred with the same number of culturable cells present initially and at the end of the second cycle again argues that true resuscitation occurred. It is highly unlikely that two cycles of growth, from a low number of culturable cells to relatively high cell numbers, would be possible under the conditions of nutrient limitation present in our study. The question of how the cells reorganize endogenous energy and nutrient reserves required for protein and peptidoglycan synthesis during this resuscitation needs to be addressed. Any residual nutrients from the initial growth medium carried over during inoculation of the microcosm should have been consumed during the first round of resuscitation. This suggests that the second round of resuscitation was undertaken by cells that can respond solely to a temperature upshift in a nutrient-free medium. In the natural environment, when the surrounding waters gradually become warmer during the spring, cells of *V. vulnificus* are thus likely to resuscitate from the nonculturable state, even if nutrient levels are low. In the presence of nutrient, they are then likely to initiate DNA replication and growth.

In the only other study reported on resuscitation of nonculturable bacteria, Roszak et al. (22) added various concentrations of heart infusion broth to nonculturable *S. enteritidis* cells. Because no total direct cells count data were presented for the cells following the nutrient addition, it is difficult to evaluate whether the true resuscitation of the

nonculturable cells occurred, as opposed to reproduction of a few residual culturable cells. In our study, nonculturable cells of *V. vulnificus* could be resuscitated to the original numbers present prior to low-temperature incubation simply by incubation at room temperature for 3 days and without nutrient addition to the samples. Only following complete resuscitation were the plate counts and total cell counts seen to increase slightly compared with the initial values. This could be due to carryover of nutrients from the initial growth medium to the nutrient-free microcosm. However, the maximum amount of nutrients in the microcosms as a result of the dilution procedure must be considerably less than 8 mg of organic carbon per liter (the amount present in VNSS). Another explanation could be cryptic growth. Postgate and Hunter (20) calculated that approximately 50 cells need to die to support growth of one cell. Calculations with the highest total number of cells used in our study could account for an increase of only $1.5 \times 10^5$ cells per ml, or only 2% of the total cell number observed. Thus, cryptic growth could not explain the increases observed in our study. A third possibility is that if a few culturable cells remained in our microcosm, an increase in plate counts from <0.1 to $2 \times 10^6$ CFU/ml would be reflected by the total (AODC) counts increasing from $2 \times 10^6$ to $4 \times 10^6$. Such a small increase might not be readily apparent. Evidence that this third possibility was not the case includes the fact that the total cell numbers did not increase until after complete resuscitation, that microscopic examination revealed no cocci present after 48 h (indicating that the cocci present prior to the temperature upshift had resuscitated to rods), and that two cycles of nonculturability and resuscitation were shown experimentally with the same number of culturable cells at the beginning and end of each cycle. It is highly unlikely that two cycles of growth from a low number of culturable cells to relatively high cell numbers would be possible under conditions of nutrient limitation.

A fourth and most likely reason for the total cell increase observed following resuscitation is that as a response to room temperature and starvation conditions for 72 h, the larger cells seen after 48 h at room temperature go through reductive cell division upon encountering the starvation conditions present in the microcosms. In a recent study by Oliver et al. (18), when *V. vulnificus* was inoculated into nutrient-free ASW microcosms and incubated at room temperatures, a significant increase in plate counts occurred, whereas the cultures at 5°C did not show an increase. Our results correspond well with these findings and suggest that reductive division was occurring in our cultures following temperature upshifts and completion of resuscitation.

Albertson et al. (1) also demonstrated that there was good correlation between the time *Vibrio* sp. strain S14 had been totally starved for nutrients before the nutrient upshift and the lag periods obtained for initiation of DNA synthesis and for an increase in cell numbers. This may also pertain to the initiation of resuscitation in nonculturable cells of *V. vulnificus*. A culture that has been at 5°C for a long time would probably need a lag period, i.e., the I phase of the cell cycle, of more than 24 h before cells could be detected on solid media.

The results obtained by adding chloramphenicol and ampicillin to resuscitating cells suggested that active protein and peptidoglycan synthesis were ongoing during all stages of resuscitation. The decrease in viability with prolonged starvation after addition of chloramphenicol resembles that found for chloramphenicol-treated, starved cells of *Vibrio* sp. strain S14 (14). The recovery of starved S14 cells was shown to include a starvation phase after the nutrient upshift but prior to biomass increase, during which time 10 proteins specific to this phase were found to be synthesized (1). An additional 11 proteins initiated in the maturation phase were synthesized also after growth had commenced. Studies aimed at identifying de novo synthesis of resuscitation specific proteins in *V. vulnificus* are now being undertaken in our laboratories. When the inhibitors were added to nonculturable cells immediately at temperature upshift or to cells that had been at room temperature for up to 24 h, resuscitation was completely inhibited. When inhibitors were added to samples that had been at room temperature for 72 h, only a fraction of the population was still dependent on protein and peptidoglycan synthesis (Fig. 4D). This coincides with the time that it took the nonculturable cells to resuscitate to their initial numbers. Cell lysis was not induced by the inhibitors, as determined by the constant number of the total cell counts. This finding suggests that nonculturable cells may develop autolysin-resistant cells similar to the response displayed by starved bacteria (15, 24).

The viable but nonculturable state could be a dual response to a temperature downshift resulting in starvation due to either lack of exogenous nutrient or a shutdown of nutrient transport systems as a result of the low temperature. However, while overlap may exist, the starvation response appears to be different from the nonculturable response, and in fact the starvation survival response may repress the latter. We have observed (18) that cells of *V. vulnificus* that are carbon starved at room temperature before temperature downshift do not become nonplateable.

Characterization on a molecular level of the processes leading to the nonculturable state, as well as of those responsible for initiating resuscitation, is of considerable interest. In *E. coli*, it has been demonstrated that a downshift in temperature, from 37 to 10°C, of an exponentially growing culture induces synthesis of 13 cold shock-specific proteins, some of which may be involved in the adaptation of the organism to growth at the lower temperature (7). Whether such proteins may be of importance to the survival of the extremely invasive human pathogen *V. vulnificus* on experiencing temperature downshifts and entering into the nonculturable state is now being examined in our laboratories.

## ACKNOWLEDGMENTS

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