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

MARK D. WHITESIDES AND JAMES D. OLIVER*

Department of Biology, University of North Carolina at Charlotte, Charlotte, North Carolina 28223-0001

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Like many other gram-negative bacteria, the human pathogen *Vibrio vulnificus* is induced into a viable but nonculturable (VBNC) state by incubation at low temperatures. The ability of any bacterium to resuscitate from this dormant state would appear to be essential if the VBNC state is truly a survival strategy. The question as to whether the culturable cells which appear following removal of the inducing stress are a result of true resuscitation or of regrowth of a few residual culturable cells has long been debated. *V. vulnificus* was examined for its ability to resuscitate from this state following a temperature upshift. Several lines of investigation, including dilution studies, determination of the time necessary for appearance of a culturable population, and the effects of nutrient on recovery, all indicated that, at least for *V. vulnificus*, true resuscitation does occur. Our studies further suggest that nutrient is in some way inhibitory to the resuscitation of cells in the VBNC state and that studies which add nutrient in an attempt to detect resuscitation are able to detect only residual culturable cells which might be present and which were not inhibited by the added nutrient.

Vibrio vulnificus is an estuarine bacterium present in temperate waters throughout the world. It is a significant human pathogen, producing potentially fatal infections following ingestion, typically of raw oysters, or through contamination of a wound (6, 10). The bacterium is easily isolated from both water and shellfish during warm months, but its isolation during cold months has proved difficult (12, 16, 20, 21). This decrease in culturability is thought to be due to the entrance of *V. vulnificus* into a viable but nonculturable (VBNC) state (11, 12, 15), as has been now shown for at least 30 other bacterial species (12). It is thought that the VBNC state may represent a survival response by bacteria exposed to potentially injurious environmental conditions (11, 27) and in the case of *V. vulnificus* is induced by incubation at temperatures below 10°C (8, 25).

Whereas cells in the VBNC state will not grow in or on nutrient media, studies in both the laboratory (9) and the field (14) have indicated that, if *V. vulnificus* is subjected to a temperature upshift prior to attempted culture, the cells are able to recover from this dormant state, become metabolically active and fully culturable, and retain virulence (13). However, whether true resuscitation of such cells occurs has long been debated. While several studies have concluded that VBNC cells do indeed leave this dormant state on removal/reversal of the inducing factor (4, 5, 9, 14, 22), others have suggested that the culturable population which appears is a result of regrowth of one or more culturable cells in the population which had not been detected when the population was assayed (2, 17, 24, 26). In most of the latter studies, nutrient in the form of bacterial medium was added to the VBNC cells in order to detect, often by the most-probable-number method, the presence of culturable cells and/or to determine if the VBNC cells could resuscitate (1, 3, 23, 24). The conclusion of these studies has generally been that only if culturable cells were present among the population of VBNC cells would a large population of culturable cells develop. However, we reasoned that, while the development of a large culturable population would indeed appear if any culturable cells were present and nutrient was added, the fact that cells in the VBNC state do not develop in or on nutrient media suggests that elevated nutrient might be lethal to VBNC cells or might at least be inhibitory to their resuscitation. If this is the case, then the addition of nutrient media to a population of VBNC cells would allow the development of a culturable population only if culturable cells were present among the VBNC cells.

To test this theory, we examined the ability of cells of *V. vulnificus* to resuscitate from the VBNC state and the effects of nutrient on this resuscitation. Studies were also conducted wherein VBNC populations, diluted to such an extent that the presence of any culturable cells would not be possible, were resuscitated. Finally, we examined the time required for culturable cells to develop from a fully nonculturable population. These studies suggest, at least in *V. vulnificus*, that true resuscitation from the VBNC state does occur but that such resuscitation is inhibited by nutrient media.

MATERIALS AND METHODS

Preparation of VBNC populations. *V. vulnificus* C7184 opaque (19) was employed in these studies. To produce VBNC populations, cells were incubated at 37°C in heart infusion (HI) broth (Difco Laboratories, Detroit, Mich.) until early logarithmic phase (optical density at 610 nm, 0.15 to 0.2; ca. 10^8 CFU/ml). Cells (1 ml) were then washed twice in artificial seawater (ASW) (25) to remove any nutrient, resuspended in 1 ml of ASW, and placed into 99 ml of ASW to form a 100-ml nutrient-free microcosm containing ca. 10^6 CFU/ml. Microcosms were immediately transferred to 5°C, and aliquots were periodically plated onto HI agar at room temperature (ca. 22°C) to determine culturability. When <0.1 CFU of culturable cells per ml was present, the population was considered nonculturable. This level of detection was achieved by filtering 10 ml of the microcosm through a 0.2-μm-pore-size filter (Nuclepore) and placing the filter onto an HI plate. Culturability was also determined by inoculating 1 ml of a nonplateable population into 1 ml of HI broth. These samples were allowed to incubate at room temperature for 25 h. To determine if the nonculturable cells were in the VBNC state, the direct viable count (DVC) method of Kogure et al. (7) was employed, with incubation of cells in the presence of 0.002% nalidixic acid and 0.025% yeast extract for ca. 12 h at room temperature. Total cell numbers were determined for the same samples following acridine orange staining (18).

Resuscitation studies. To determine if the appearance of culturable cells following a temperature upshift of the microcosm was a result of true resuscitation of the VBNC cells present, as opposed to regrowth of a few culturable but undetected cells, 10 individual 1-ml samples of the VBNC (<0.1 CFU/ml) microcosm were removed from 5°C and allowed to incubate at room temperature for 24 h. Similarly, dilutions in ASW (to a maximum of 10^{-3} of the VBNC population) were also made, and 1-ml volumes were allowed to incubate over-

* Corresponding author. E-mail: jdoliver@unccvm.uncc.edu.

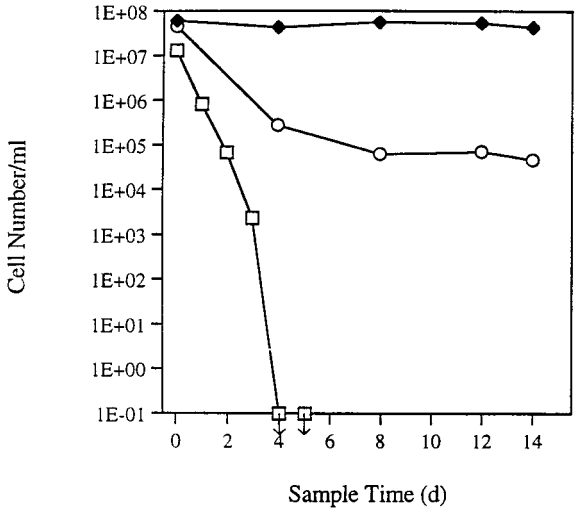

FIG. 1. Entry of *V. vulnificus* into the viable but nonculturable state in an ASW microcosm at 5°C. Shown are plate counts (□) on HI agar in CFU per milliliter, total cell counts (◆) by the acridine orange staining method, and direct viable counts (○) by the substrate responsiveness method of Kogure et al. (3). d, day.

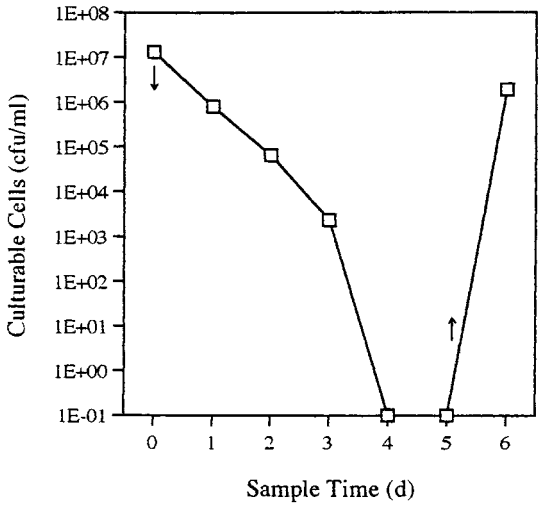

FIG. 2. Loss of culturability of *V. vulnificus* in an ASW microcosm incubated at 5°C (downward arrow) followed by recovery of culturability after a temperature increase (upward arrow) to ca. 22°C for 24 h. d, day.

night at room temperature. Culturability of these samples was determined following plating on HI agar. This study was repeated three times. In a separate study, a VBNC (<0.1 CFU/ml) microcosm was removed from 5°C to room temperature, and the population was diluted and plated onto HI agar at hourly intervals to determine the time required for the appearance of culturable cells.

Effect of HI broth on resuscitation of VBNC cells. Aliquots of a microcosm containing VBNC cells (<0.1 CFU/ml) which had been shown to contain cells capable of resuscitation from the VBNC state by transfer from 5°C to room temperature were combined with HI broth at a 1:1 ratio. Cells were then allowed to incubate in the presence of this elevated nutrient at room temperature for various times. Cells were then centrifuged and washed in ASW to remove all nutrient. Sterile ASW (1 ml) was then added to the cells, which were then incubated at room temperature for an additional 24 h. Culturability was subsequently determined by plating onto HI agar.

RESULTS AND DISCUSSION

Figure 1 shows the response of *V. vulnificus* following its incubation in ASW at 5°C. Total cell counts remained constant throughout the 14-day period, while plate counts rapidly declined to undetectable levels (<0.1 CFU/ml) within 4 to 6 days. DVCs, on the other hand, declined to ca. 10^5 active cells and remained fairly constant at this level. These results are typical of the several microcosms prepared for the studies we report here and for studies we have previously reported for *V. vulnificus* under these conditions.

Cells from such microcosms placed into HI broth at room temperature for 24 h did not produce turbidity, nor did such treatment result in cells which subsequently give rise to colonies when plated onto HI agar. Indeed, we observed that VBNC cells of *V. vulnificus* plated onto HI agar and incubated in a moist chamber for 30 days at room temperature do not result in colony formation. In contrast, when cells from our nutrient-free ASW microcosms were subjected to a simple temperature upshift (to ca. 22°C) for 24 h, resultant plating onto HI agar routinely provided culturable populations of ca. 10^6 CFU/ml (Fig. 2). This observation is similar to that we previously reported (9), although the strains and culture conditions differed in that study.

As resuscitation of VBNC cells was shown not to occur in liquid or solid growth medium, it was considered that elevated nutrient might be toxic in some manner to cells in this state. However, when VBNC cells were incubated in the presence of HI broth for periods of up to 12 h and subsequently allowed to incubate in nutrient-free ASW for an additional 24 h, no decrease in the extent of resuscitation was observed compared to control cells allowed to resuscitate in ASW without prior exposure to the elevated nutrient (Fig. 3). Why resuscitation appears to occur only when little or no nutrient is present is not understood, although this study suggests that HI broth is not, in itself, toxic to cells in the VBNC state. It is also important to note that, regardless of the duration for which the VBNC cells were present in this high level of nutrient, the number of culturable cells which were ultimately present did not exceed that of control cells which were resuscitated at room temperature but without being exposed to the added nutrient. If culturable cells had been present among the VBNC cells, a population approaching 10^8 CFU/ml or greater would have been expected following these conditions of incubation. This

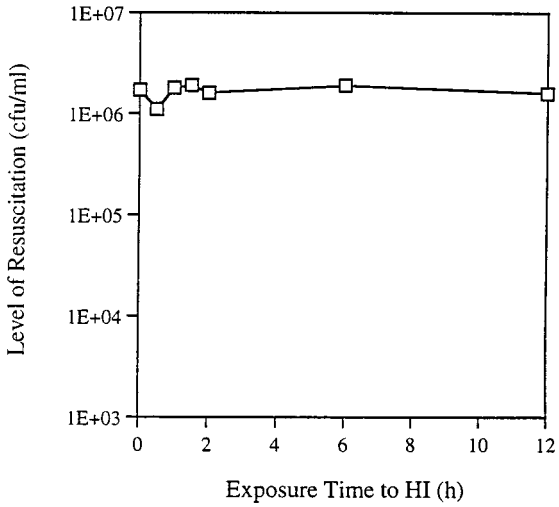

FIG. 3. Effect of elevated nutrient on ability of *V. vulnificus* to resuscitate. Cells were added at a 1:1 ratio to HI broth and incubated for periods up to 12 h, at which time the nutrient was removed and the cells were resuspended in nutrient-free ASW. After an additional 24-h incubation at room temperature, cells were plated onto HI agar.

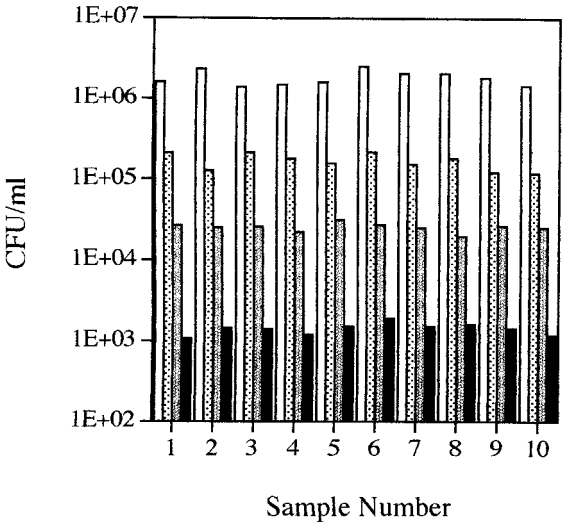

FIG. 4. Resuscitation of *V. vulnificus* from the VBNC state (<0.1 CFU/ml) as observed in 10 individual 1-ml aliquots of an undiluted sample (□) and of samples diluted 10^{-1} (▨), 10^{-2} (▩), and 10^{-3} (■). Resuscitation was at room temperature for 24 h, with plating onto HI agar.

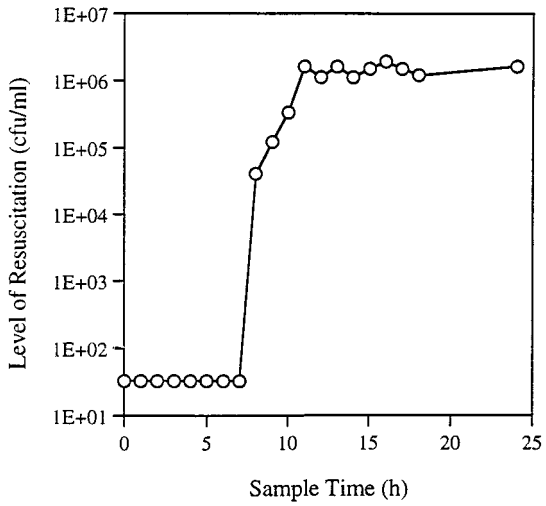

FIG. 5. Time required for resuscitation of VBNC *V. vulnificus* cells. Cells from a VBNC microcosm (<3.3×10^1 CFU/ml) were shifted to room temperature, and aliquots were removed at hourly intervals and plated onto HI agar.

observation argues that no culturable cells were present in the microcosms.

To further examine whether regrowth of undetected culturable cells, as opposed to true resuscitation of VBNC cells, might account for the appearance of the large culturable populations routinely observed following a temperature upshift in nutrient-free ASW, 10 aliquots of 1 ml each of nonculturable (<0.1 CFU/ml) cells were incubated for 24 h at room temperature and plated onto HI agar. Every time this multitube study was conducted, each of the 10 subsamples was observed to resuscitate to approximately the same level (ca. 2×10^6 CFU/ml). This experiment was again repeated, but with dilutions in ASW of up to 10^{-3}. As is evident from Fig. 4, resuscitation again occurred in each tube, with final culturable populations reaching levels reflecting the dilution applied. Such studies were conducted as many as 25 days after the cells had entered the VBNC state (<0.1 CFU/ml), always with the same results. These tubes contained no exogenous nutrient, and in the case of the greatest (10^{-3}) dilution, each of the 10 tubes would contain <0.0001 CFU per tube, precluding the possibility of any culturable cells being present and growing. Thus, the appearance of culturable cells following the temperature upshift must have been due to true resuscitation of the VBNC population and not to regrowth of undetected culturable cells.

Several preliminary experiments with different VBNC microcosms indicated that, following a temperature upshift from 5°C to room temperature, culturable cells began to appear considerably before the 24-h sample period we had routinely employed for our platings. Consequently, we sampled a VBNC microcosm more frequently, with samples being taken at hourly intervals following the temperature upshift. In this study, no culturable cells could be detected (<3.3×10^1 CFU/ml) while total cell numbers were 6.3×10^6 and DVC values were 1.0×10^6. Culturable cells first appeared at 8 h after the temperature upshift (Fig. 5). Initial numbers of culturable cells were at 5×10^4, with levels rapidly increasing to a maximum within 3 additional hours of room temperature incubation. As the culturable cell density at 7 h was below our limit of detection (<3.3×10^1 CFU/ml) but reached 5×10^4 by 8 h, it would appear that >10 generations would have had to occur during this 1-h period if the increase had been due to culturable cells. Such an increase would require a generation time of ca. 6 min, a rate clearly impossible following room temperature incubation in a nonaerated ASW solution free of any added nutrient. A rate of resuscitation far greater than the division rate of *Micrococcus luteus* was also observed by Kaprelyants and Kell (4). These results again argue strongly that true resuscitation of a VBNC population occurred, and not regrowth of culturable cells.

In conclusion, various approaches were taken to examine whether true resuscitation of VBNC cells of *V. vulnificus* occurs following a temperature upshift. All of our studies argue strongly that true resuscitation occurs. We do not yet know why cells in the VBNC state will not grow in or on the bacterial media routinely employed for their culture. Our studies suggest that elevated nutrient (e.g., full-strength medium) is in some way inhibitory to cell division in VBNC populations, but the mechanism of this inhibition is not yet clear. However, the potential significance of the VBNC state in public health microbiology, in the use of bacteria as indicator organisms, and in our ability to monitor genetically modified bacteria released into the environment all argue for increased study of this survival response.

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